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STUDIES ON THE MECHANISM OF THE GROWTH-INHIBITORY ACTION OF FUSARINIC ACID ON PLANTS[1]

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(Received for publication, October 14, 1953)

In 1934 Yabuta (*1*) isolated a crystalline toxic substance, possessing a marked growth-inhibitory action on rice seedlings (Photo. 1), from the culture liquids of *Gibberella Fujikuroi Wr.*, the causative mould of the "BAKANAE" disease of rice plants, and named this substance fusarinic acid (FA).[2] The chemical structure of FA was confirmed also by him to be identical with β'-*n*-butylpyridine-α-carboxylic acid.

The present communication[3] deals with the mechanism of the

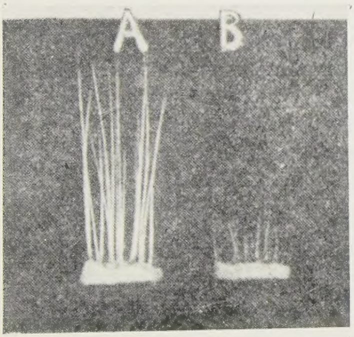

Photo. 1. Rice seedlings cultured for two weeks at 30°
A: Distilled water (Control test)
B: 0.01% fusarinic acid solution

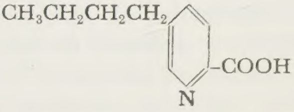

Diagram 1
Fuarinic acid

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[1] This investigation was supported by a grant in aid for fundamental scientific research from the Ministry of Education, in Japan.

[2] The following abbreviation will be used in this paper:
FA, fusarinic acid; PA, α-picolinic acid; QA, quinolinic acid; 8HQ, 8-hydroxyquinoline; PCA, α-picoline-γ-carboxylic acid.

[3] This is an out-look of the original reports: *J. Agr. Chem. Soc. Japan*, **26**, 223, 295, 298, 345, 349 (1952); **27**, 144, 147, 159, 245, 249, 302 (1953).

growth-inhibitory action of FA on plants.

EXPERIMENTAL

Preparation of the Materials—FA was obtained following the method of Yabuta (*1*) and the final product, recrystallized twice from ligroin, was in the form of colorless plate crystal, m. p. 108°.

PA was prepared in our laboratory from α-picoline (PA) by the method of Mende (*2*), QA from quinoline by the method of Camps (*3*), and PCA from pyruvic acid by the method of Böttingen (*4*).

Crystalline catalase was isolated from cattle liver by the method of Shirakawa (*5*); catalase activity was approximately 30,000.

Cultivation of Test Rice Seedlings—Test rice grains were kept on absorbent cotton in a glass dish (diameter 4.5 cm., depth 3.3 cm.) which contains 20 ml. of the inhibitor solution (pH 5.5~5.8), and distilled water, equivalent to the evaporated water, was supplied every day.

Estimation of the Respiration Value of Test Rice Seedlings—The respiration value was determined by Warburg's manometer, holding a special vessel (Fig. 1).

Determinations of the Type and the Intensity of the Inhibitory Action of the Inhibitor on the Respiration of Rice Seedlings—The experimental data of the respiration-hindrance of rice seedlings, affected by the inhibitory action of the inhibitor, were treated by the method of analysis of covariance and the following regression equation of correlation coefficient ranging from 0.96 to 0.98 was obtained between the hindrance coefficient[4)] (H) of the respiration and the concentration (G) of the inhibitor: $H = n \log G - n \log \phi + 0.5$, where n represents the type of the inhibitory action and ϕ the molality of the inhibitor for 50 per cent inhibition of the respiration, namely a reciprocal value of the intensity of the inhibitory action. The concentrations of the inhibitor used for the establishment of the regression equation are as follows: 0.00014, 0.00028, 0.00042, 0.00056 and 0.00112 *M*.

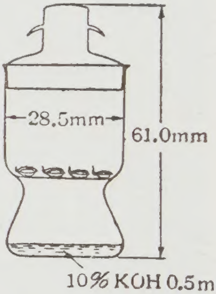

FIG. Special vessel

[4)] The hindrance coefficient is calculated by the formula: 1–c/C, where represents the respiration-value, etc. of the rice seedling cultured under the injurious effect of the inhibitor and C that of the control.

Estimations of the Catalase and Peroxidase Powers of Test Rice Seedlings,—Ten test rice seedlings cultured with the inhibitor solution were peeled, mashed well, mixed with 25 ml. of $M/15$ K_2HPO_4 (pH 7.2) and centrifuged. A portion of 10 ml. of the supernatant solution was submitted to the estimation of catalase power by the method of Hennichs (*6*) and a portion of 6 ml. of the solution to the estimation of peroxidase power by the method of Bach-Zubcowa (*7*).

Determinations of the Type and the Intensity of the Inhibitory Action of the Inhibitor on Each of the Catalase Power and the Peroxidase Power of Rice Seedlings—The following regression equation of correlation coefficient ranging from 0.95 to 0.98, was obtained between the hindrance coefficient (H) of each of the catalase power and the peroxidase power of the test rice seedling and the concentration (G) of the inhibitor: $\log H/(1-H)=n\log G-n\log\phi$, where the definitions of n and ϕ, and the concentrations of the inhibitor used for the establishment of the regression equation are the same as mentioned in the case of the respiration.

Estimation of the Contents of Ferric Ion in the 100 Rice Seedlings Cultured under the Injurious Effect of the Inhibitor—The estimation was carried out by the aid of polarography. One hundred of the rice seedlings cultured with the inhibitor solution for 4 days at 30° were peeled, mashed well and extracted with 30 ml. of the distilled water (pH 4.0), acidified with H_2SO_4, and centrifuged. A 5 ml.-portion of the supernatant solution was mixed with 1 drop of gelatin solution and submitted to the polarography. The contents of ferric ion in the 100 test rice seedlings were calculated by the formula: $Fe^{+++}(\text{mg.})=0.13\,\text{mg.}\times A/(C-B)\times 30/5$, where A represents the wave-height of the polarogram of ferric ion in the extractive of test rice seedlings, B that in the extractive of the control seedlings and C that in the extractive of the control mixed with 0.13 mg. of ferric ion.

Elimination Test of the Inhibitory Action of FA on the Respiration, on the Catalase Power and on the Peroxidase Power of Rice Seedlings by the Addition of the Suitable Metal Ions—The study on the elimination of the inhibitory action of FA by the addition of metal ions was accomplished by the estimation of ϕ/ϕ' value, where ϕ* and ϕ'* respectively represent the reciprocal values of the inhibitory actions of FA and FA plus metal ions being calculated by the corresponding regression equations.

The Chelation Intensities of the Ferric and Cupric Salts of Each of the Inhibitors—The chelation intensity was calculated by the formula (H−h)/

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* Molality of the inhibitor for 50 per cent inhibition.

H, where H represents the wave-hight of the polarogram of ferric ion in the standard solution (A) or that of cupric ion in the solution (B), and h represents that of ferric ion in the test solution (a) or that of cupric ion in the solution (b).

Standard solution A: 0.0028 *N* $Fe_2(SO_4)_3$, 2 ml. + distilled water, 6 ml.
,, ,, B: 0.0028 *N* $CuSO_4$, 2 ml. + 1 *N* NH_4Cl, 2 ml. + 1 *N* NH_4OH, 2 ml. + distilled water, 2 ml.
Test solution a: 0.0028 *N* $Fe_2(SO_4)_3$, 2 ml. + 0.0028 *N* inhibitor, 2 ml. + distilled water, 4 ml.
,, ,, b: 0.0028 *N* $CuSO_4$, 2 ml. + 0.0028 *N* inhibitor, 2 ml. + 1 *N* NH_4Cl 2 ml. + 1 *N* NH_4OH, 2 ml.

The Chelation Intensity of FA with Each of the Metal Ions (Fe^{+++}, Cu^{++}, Cd^{++}, Ni^{++} *and* Co^{++})—The chelation intensity was calculated by the formula, $(H-h)/H$, where H represents the wave-height of polarogram of the metal ion in the standard solution and h represents that of the metal ion in the test solution.

Standard solution: 0.0028 *N* metal-salt solution 2 ml. + distilled water 2 ml.
Test solution : 0.0028 *N* metal-salt solution 2 ml. + 0.0028 *N* FA solution. 2 ml.

As metal salts $Fe_2(SO_4)_3$, $CuSO_4$, $CdCl_2$ $NiCl_2$ and $Co(NO_3)_2$ were used for the experiment.

RESULTS AND DISCUSSION

The Effect of FA on the Growth of Rice Seedlings—The characteristic

TABLE I

Root Formation and Sprouting of the Rice Seedling in the Initial Growing Stage under the Injurious Effect of Fusarinic Acid

Concentration of fusarinic acid in the culture solution	Percentage of rooting. Sample size =20	Average of root -lengths.	S.D.	Percentage of germination. Sample size =20	Average of coleoptile -lengths.	S.D.
%	%	*mm.*	*mm.*	%	*mm.*	*mm.*
Distilled water (Control test)	85	9.7	5.1	75	2.6	0.7
0.001	95	10.7	3.2	95	2.7	0.7
0.0025	75	6.6	2.5	90	2.9	1.0
0.005	5	3.0	—	100	3.2	9.9
0.0075	10	1.5	—	80	3.3	0.8

S.D. Standard deviation

In each test 20 rice grains were kept for 50 hours at 30° in a glass dish on absorbent cotton.

symptom of the rice seedling in the initial germination stage affected by the injurious action of FA is that the root-formation is strongly disturbed, whereas the coleoptile does not suffer almost any inhibition (Table I). This symptom is quite similar to that observed on the rice seedling which germinates in the oxygen free atmosphere (Photo. 2). This observation led us to an assumption that the growth-inhibitory action of FA should be due to the hindrance of the respiration of plants. This assumption is further supported by the following observation.

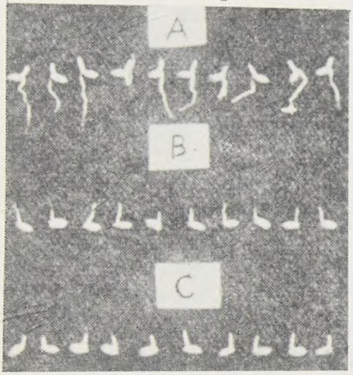

Photo. 2. The test rice grains (Norin No. 1) were kept in glass dish on wet absorbent cotton for 4 days at 30°. A: Rice seedlings cultured with distilled water. B: Rice seedlings cultured with distilled water in the oxygen free atmosphere (N_2 gas). C: Rice seedlings cultured with 0.02% fusarinic acid solution.

The Effect of FA on the Respiration, on the Catalase and Peroxidase Powers of Rice Seedlings—As shown in Table II FA and PA possess similarly the remarkable inhibitory actions on the respiration of rice plants. As comprehended by the chemical structures, FA and PA are able to form the stable inner metal complex salts, each containing a 5-membered coordinate link, namely the chelate ring which is steadied by the resonance effect. The chelate rings of the inner metal comples salts of FA and PA develope colors as chelate rings generally do so, the cupric salts both giving blue colors and the ferric salts yellow. Of the several metal complex salts of FA the ferric salt possesses a especially marked chelation intensity (Table III). From these facts the inhibitory action of FA can be explained as follows: in plant-tissues FA tightly ctches the metal ions, such as Fe^{+++}, Cu^{++}, *etc.*, by the chelation mechanism and checks the formation of metal containing enzymes, especially Fe-porphyrin oxidases, consequently depressing the respiration of plants.

This view is supported by the results presented in Table IV, V and VI which demonstrate that the contents of Fe^{+++}-ion* and Fe-porphyrin oxidases in the rice seedling cultured under the injurious effect of FA remarkably diminish as compared with those contained in the control and then the hindrance coefficient of the respiration and that of the

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* Rice plants contain Fe^{+++}-ion abundantly compared with other metal ions (*8*).

TABLE II

Respiration Value of the Rice Seedling (Norin No. 1) Cultured under the Injurious Effect of Fusarinic Acid or α-Picolinic Acid

Concentration of the inhibitor in the culture solution	Respiration value calculated as net oxygen consumption in cu.mm. per 10 seedlings per 30 minutes at 30°.	
	Fusarinic acid	α-Picolinic acid
Distilled water (Control test)	*cu.mm.* 124.5, 127.0, 130.5 130.0	*cu.mm.* 124.5, 127.0, 130.5 130.0
0.00014M (0.0025 %)	155.0, 121.5, 117.3 128.5	124.0, 123.3, 119.0 133.2
0.00028M (0.005 %)	93.1, 98.5, 101.0 104.5	111.2, 114.0, 116.5 119.2
0.00042M (0.0075 %)	80.0, 81.0, 86.3 89.1	88.8, 92.0, 90.0 97.1
0.00056M (0.01 %)	52.5, 60.4, 62.7 64.3	59.8, 65.5, 69.5 77.0
0.00112M (0.02 %)	51.9, 55.0, 54.3 57.7	51.5, 57.7, 60.0 62.4

Gas in the vessel: air

Rice grains were cultured on absorbent cotton with the inhibitor solution (see Experimental) for 4 days at 30° and submitted to the respiration test.

TABLE III

The Chelation Intensity of Fusarinic Acid with Each of the Metal Ions

	Fe^{+++}	Cu^{++}	Cd^{++}	Ni^{++}	Co^{++}
Chelation intensity of fusarinic acid with metal ion (pH 4.0)	0.88	0.60	0.52	0.43	0.28

$Fe_2(SO_4)_3$, $CuSO_4$, $CdCl_2$ and $Co(NO_3)_2$ have been used for the experiment.

catalase power or the peroxidase power of the rice seedling germinated under the injurious effect of FA correlates with a very high confidence coefficient. This fact means that the respiration-hindrance of the seedling caused by the injurious effect of FA is chiefly due to the hindrance of the Fe-porphyrin oxidase system.

TABLE IV

The Contents of Ferric Ion in the 100 Rice Seedlings Cultured under the Effect of Fusarinic Acid, α-Picolinic Acid or 8-Hydroxyquinoline

Inhibitor	FA	PA	8HQ	Control
Concentration, *M*	0.0056 (=0.01%)	0.0056	0.0056	Distilled water
The contents of ferric ion in 100 rice seedlings, *mg.*	0.42	0.54	0.24	0.75

Rice grains were cultured with the inhibitor-solution on absorbent cotton for 4 days at 30°.

The contents of ferric ion in the rice seedlings, cultured under the effect of FA, PA or 8HQ, are remarkably diminished as compared with those in the control rice seedlings in proportion to the chelation intensity of the inhibitor (see Table XV).

TABLE V

Inhibitory Effects of Fusarinic Acid and α-Picolinic Acid on the Catalase Power of the Rice Seedlings

Concentration of the inhibitor.	Catalase power*	
	Fusarinic acid	α-Picolinic acid
Distilled water (Control test)	(Average of 3 values) 5.95 *ml.*	(Average of 3 values) 5.95 *ml.*
0.00014 *M*	5.59	5.85
0.00028 *M*	4.78	4.50
0.00042 *M*	4.49	4.69
0.00056 *M*	3.48	3.53
0.00112 *M*	3.01	3.24

* Catalase power is indicated as ml. of $N/20$ $KMnO_4$ when estimated by the method of Hennichs (*6*).

Rice grains were cultured on absorbent cotton with the inhibitor-solution for 4 days at 30° and submitted to the test of catalase power.

The Elimination of the Inhibitory Action of FA by the Addition of Suitable Metal Ions—The role of the chelation mechanism as a principal toxic factor of FA is shown by the following observations.

As shown in Table VII the inhibitory actions of FA on the respiration and on the Fe-porphyrin oxidase system of rice seedlings are both

TABLE VI

Inhibitory Effects of Fusarinic Acid and α-Picolinic Acid on the Peroxidase Power of the Rice Seedling

Concentration of the inhibitor	Peroxidase power*	
	Fusarinic acid	α-Picolinic acid
Distilled wate (Control test)	(Average of 3 values) 3.17 *mg.*	(Average of 3 values) 3.17 *mg.*
0.00014 *M*	1.95	2.27
0.00028 *M*	1.75	1.95
0.00042 *M*	1.38	1.66
0.00056 *M*	1.05	1.35
0.00112 *M*	0.64	0.88

* Peroxidase power is represented by mg. of guaiacol, oxidized by the peroxidase.

The condition of the cultivation of the seedlings is the same as in Table V.

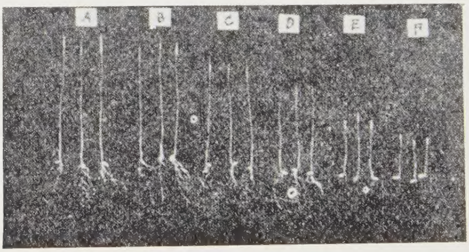


Photo. 3. Elimination of the growth-inhibitory action of fusarinic acid on rice seedlings by the addition of metal ions.

A: Distilled water. (Control test)
B: 0.0042 *N* $Fe_2(SO_4)_3+CuSO_4+MnSO_4{}^*+MgSO_4{}^*$ (Blank test)
C: 0.0056 *N* (0.01%) FA+0.0042 *N* $Fe_2(SO_4)_3+CuSO_4{}^*+MnSO_4{}^*+MgSO_4{}^*$
D: 0.0056 *N* FA+0.0056 *N* $Fe_2(SO_4)_3$
E: 0.0056 *N* FA+0.0056 *N* $CuSO_4$
F: 0.0056 *N* FA
* 6/1,000,000 in weight ratio

eliminated generally by 50 per cent by the addition of an equivalent of $Fe_2(SO_4)_3$ and then nearly all by the addition of ferric salt together with a slight amount of $CuSO_4$, $MnSO_4$ and $MgSO_4$ to the culture medium (Photo. 3).

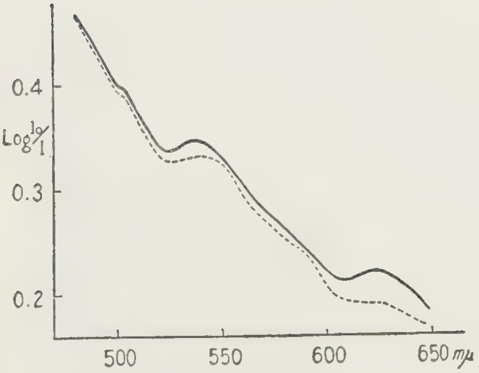
Inhibitory Actions of Some Pyridine Derivatives on the Plant Growth—It has also been found that of the substances, possessing their chemical structures related to FA, pyridine, alkyl-pyridine or nicotinic acid shows a non-inhibitory action on rice seedlings at a low concentration below 0.1 per cent and on the other hand PA, iso-nicotinic acid and their alkyl derivatives, forming the stable inner metal complex salts by the chelation mechanism, all give remarkable growth-inhibitory actions in proportion to their chelation intensities (Photo. 4, Table VIII and Diagram 2).

The Inhibitory Action of FA on the Function of the Crystalline Catalase—It is also noteworthy that FA not only suppresses the formation of Fe-porphyrin oxidase system in plant tissues, but also the activity of the purified Fe-porphyrin oxidase. From the following experimental results it may be suggested that FA must coordinately combine with the hemin-Fe in the catalase molecule by the chelation mechanism.

The experiments have been undertaken with the purified crystalline catalase.

(a) On addition of FA the absorption spectrum of the catalase suffers a change distinguished by the vanishing of λ max=627 mμ which relates to the hemin-Fe in the catalase molecule (Graph 1).

(b) On addition of FA the polarogram (Graph 2) and the redox-potential of the catalase remarkably fall (Table IX). The fall of the redox-potential of the Fe-porphyrin oxidase makes the hydrogen-transfer



Graph 1. Absorption spectrums of catalase and FA-catalase measured by aid of Beckmann-spectrophotometer.
—— Catalase
---- FA-catalase
(Catalase solution 2ml.+0.7% FA solution 3 ml.+M/10 phosphate buffer (pH 6.9) 1ml.) pH 6.8, Temperature: 11°C.

TABLE VII

Elimination of the Inhibitory Action of Fusarinic Acid on the Respiration and on the Fe-porphyrin Oxidase System of Rice Seedlings by the Addition of Metal Ions

Hindrance of the Respiration

Test solution	Average of ϕ (or ϕ') values and confidence limits of population mean. Sample size=4, $\alpha=0.05$	ϕ/ϕ'*
(a) FA	9.000755±0.000041 (ϕ)	
(b) Fa+Cu^{++}	0.001102±0.000080 (ϕ')	0.68
(c) FA+Fe^{+++}	0.001480±0.000120 (ϕ')	0.51
(d) FA+Fe^{+++}, Cu^{++}, Mn^{++}	0.004610±0.00062 (ϕ')	0.16
(e) Fa+Fe^{+++}, Cu^{++}, Mn^{++}, Mg^{++}	0.00500 ±0.00070 (ϕ')	0.15

Hindrance of the Catalase Power

Test solution	Average of ϕ (or ϕ') values and confidence limits of population mean. Sample size=4, $\alpha=0.05$	ϕ/ϕ'*
(a) FA	0.00100±0.000071 (ϕ)	
(b) FA+Cu^{++}	0.00161±0.00011 (ϕ')	0.62
(c) FA+Fe^{+++}	0.00229±0.00018 (ϕ')	0.43
(d) FA+Fe^{+++}, Cu^{++}, Mn^{++}	0.00678±0.00078 (ϕ')	0.22
(e) FA+Fe^{+++}, Cu^{++}, Mn^{++}, Mg^{++}	0.00576±0.00083 (ϕ')	0.17

Hindrance of the Peroxidase Power

Test solution	Average of ϕ (or ϕ') values and confidence limits of population mean. Sample size=4, $\alpha=0.05$	ϕ/ϕ'*
(a) FA	0.000676±0.000032 (ϕ)	
(b) FA+Cu^{++}	0.000930±0.000038 (ϕ')	0.72
(c) FA+Fe^{+++}	0.000934±0.000041 (ϕ')	0.68
(d) FA+Fe^{+++}, Cu^{++}, Mn^{++}	0.00319 ±0.00020 (ϕ')	0.21
(e) FA+Fe^{+++}, Cu^{++}, Mn^{++}, Mg^{++}	0.00329 ±0.00018 (ϕ')	0.17

α: Level of significance. ϕ (or ϕ'): molality of the inhibitor for 50 per cent inhibition.

* Ratio of the inhibitory action of FA+metal ions to that of FA. Test rice grains were cultured with the test solution of each concentration** of the inhibitor for 5 days at 30° and submitted to the test for the estimation of each hindrance coefficient of respiration, catalase power and peroxidase power. In all cases the regression equations were established 4 times in parallel.

** (a) 0.00014, 0.00028, 0.00042, 0.00056 and 0.00112 *M* of fusarinic acid.

Photo. 4. Rice seedlings (Norin No. 1) cultured for two weeks at 30° in glass dish on wet absorbent cotton with 0.00056 *M* solution of the substance, possessing the chemical structure related to fusarinic acid.

A: Distilled water B: FA C: PA D: PCA
E: Isonicotinic acid F: QA G: Uvitonic acid
H: Pyridine I: α-Picoline J: β-Picoline

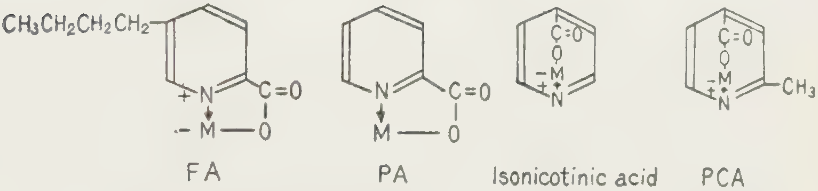


Diagram 2. Chelate structures of the inner metal complex salts of FA, PA, isonicotinic acid and PCA. M: Metal

in the respiration enzyme system very difficulty.

(c) The inhibitory action of FA on the catalase activity is perfectly eliminated on addition of Fe^{+++} ion, on this point FA is essentially different from the other catalase poisons, such as NaCN, phenol and hydrazine (Table X, Graph 3). This finding suggests a competition of the hemin-Fe with free Fe^{+++} ion for the same coordinate linkage to FA. From the Table X it has also been found that the joint of CN-catalase and phenol-catalase dissociate in the precipitation process and

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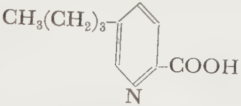
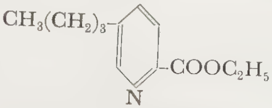
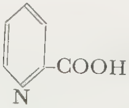
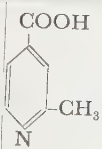
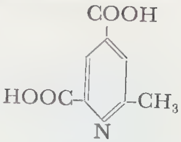
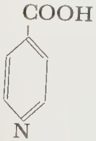
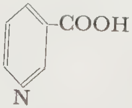
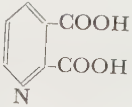
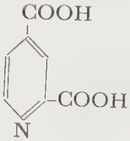
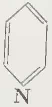
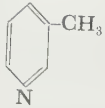
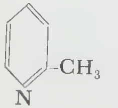
(b) To each of (a) respectively an equivalent of $CuSO_4$ was added.
(c) To each of (a) respectively an epuivalent of $Fe_2(SO_4)_3$ was added.
(d) To each of (a) 3/4 equivalent of $Fe_2(SO_4)_3$ and respective 6/1000,000 (weight ratio) of $CuSO_4$ and $MnSO_4$ were added.
(e) To each of (d) furthermore 6/1000,000 of $MgSO_4$ was added.

Notes. The data for the experiment (blank test), in which it has been confirmed that the metal salts themselves of the above mentioned concentrations have no effect (positive nor negative) on the respiration and on the Fe-porphyrin oxidase power of rice seedlings, are omitted to preserve the space of paper.

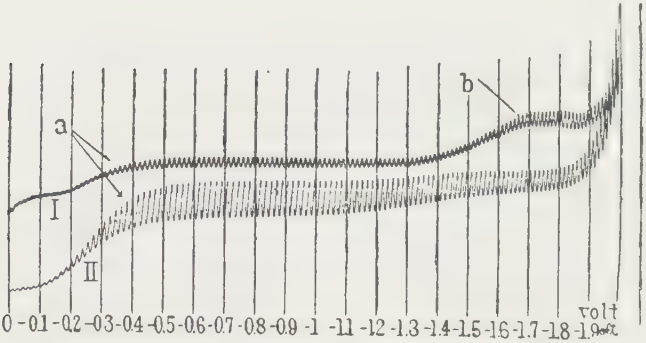
TABLE VIII

Growth-inhibitory Actions of the Substances, Possessing Their Chemical Structures Related to Fusarinic Acid, on Plants

Substance	 Fusarinic acid	 Fusarinic acid-ethylester	 α-Picolinic acid	 α-Picoline-γ-carboxylic acid	 Uvitonic acid
Growth-inhibitory action on rice-seedlings.	Very strong	Very weak	Very strong	Strong	Very weak

Substance	 Isonicotinic acid	 Nicotinic acid	 Quinolinic acid	 Lutidinic acid	 Pyrydine	 β-Picoline	 α-Picoline
Growth-inhibitory action on rice-seedlings.	Strong	—	—	—	—	—	—

—: A non-inhibitory action at the concentration below 0.1 per cent.



Graph 2. Polarograms of catalase and catalase+FA. Sensitivity: 1/5, pH 6.0 I: Catalase+FA II: Catalase a: Polarogram of porphyrin-Fe in the catalase molecule. b: Polarogram of FA.

TABLE IX

Variation of the Redox-Potential of Catalase by the Action of Fusarinic Acid.

*Catalase solution	Distilled water	Corrected redox-potential	Fusarinic acid solution 0.05%	Corrected redox-potential	(Test value)—(Blank-test value)
	(Blank-test)		(Test)		
10 *ml*	+ 0 *ml.*	261 *mv.*	+ 0 *ml.*	261 *mv.*	0 *mv.*
10	+ 2	265	+ 2	174	81
10	+ 4	255	+ 4	158	97
10	+ 7	252	+ 7	153	99
10	+10	245	+10	148	97

* Crystalline catalase has been utilized. The estimated redox-potential was converted into those of pH 6.0 state.

on the other hand that of FA-catalase and hydrazine-catalase do not dissociate in the same process. Pyridine which is the nucleus of FA shows non-inhibitory action on catalase activity.

(d) FA becomes readily non-inhibitory on the catalase activity by the esterification of its carboxyl group (Table XI). This fact evidently demonstrates that the inhibitory action of FA is based on the function of carboxyl group in the molecule namely on the chelation mechanism.

(e) On addition of the purified crystalline catalase, FA solution

TABLE X

Comparison of the Inhibitory Action of Fusarinic Acid on Catalase Activity with Those of Representative Catalase Inhibitors such as NaCN, Phenol and Hydrazine

System	Catalase activity**	
	Test A	Test B
Catalase solution* (Control test)	1.70	1.71
,, +FA	0.89	0.84
,, +FA+$Fe_2(SO_4)_3$	1.33	1.49
,, +$Fe_2(SO_4)_3$	1.45	1.61
,, +NaCN	0.73	1.41
,, +NaCN+$Fe_2(SO_4)_3$	0.88	
,, +Hydrazine sulphate	0.80	0.88
,, + ,, +$Fe_2(SO_4)_3$	0.81	
,, +Phenol	0.70	1.58
,, + ,, +$Fe_2(SO_4)_3$	0.77	
,, +Pyridine	1.60	

* Crystalline catalase has been utilized.

** Catalase activity is represented with *ml.* of $N/50$ $KMnO_4$, equivalent to the H_2O_2 which was decomposed by the catalase.

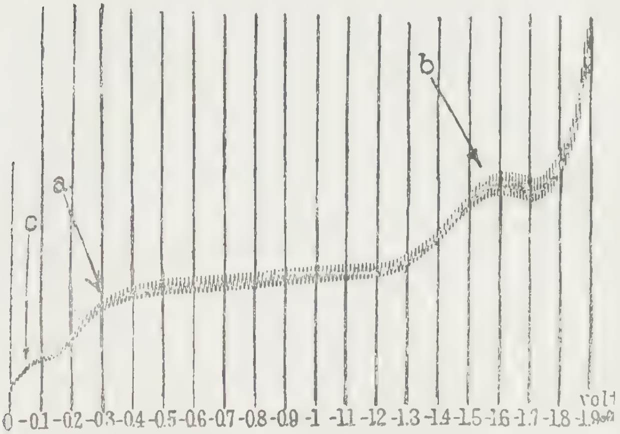
Test A: To 5 *ml.* of catalase solution were added 5 *ml.* of the inhibitor-solution (0.0028 M) and either 5 *ml.* of distilled water or 5 *ml.* of 0.0028 N $Fe_2(SO_4)_3$ solution. In the case of the control test 5 *ml.* of catalase solution was added to 10 *ml.* of distilled water. A 5 *ml.*-aliquot of this mixture was pipetted out and submitted to the test for catalase activity.

Test B: The above mentioned mixture (15 *ml.*) was mixed with 15 *ml.* of cold acetone and centrifuged– after the decantation of the supernatant solution the precipitate of the catalase was dissolved in 15 *ml.* of distilled water and then a 5 *ml.* aliquot of this solution was submitted to the test.

develops yellow color as the ferric complex salt of FA does so.

Non-inhibitory Action of FA on the No-metal Containing Enzymes– On the other hand the activities of the enzymes, in their compositions no metal participating, for example dehydrogenase, amylase, *etc.* are not inhibited by FA.

Non-antagonistic Action of Cysteine with FA—A fact that the growth-inhibitory action of FA on plants is never diminished on addition of cysteine suggests that FA may not inhibit SH-enzyme.



Graph 3. Polarogram of catalase $+FA+Fe^{+++}$

Flimination of the effect of FA on the polarogram of porphyrin-Fe in the catalase molecule by the addition of Fe^{+++} ion. (see Graph 2)

a: Polarogram of porphyrin-Fe in the catalase molecule.
b: ,, of FA.
c: ,, of $Fe.^{+++}$

Table XI

Disappearance of the Inhibitory Effect of Fusarinic Acid on the Catalase Activity by the Esterification of the Carboxyl-Group

Reaction system	Catalase activity in ml. of $N/20$ $KMnO_4$. (Average in the 3 values obtained in parallel.)
Catalase solution 10 ml + distilled water 10 ml. (Control test)	*ml.* 5.60
Catalase solution 10 ml. + 0.02% FAE 10 ml.	5.57
Catalase solution 10 ml. + 0.04% FAE 10 ml.	5.50

FAE: Fusarinic acid ethylester

The Elucidation of the Role of N-Butyl Side Chain of FA in Relation to the Inhibitory Action—For the purpose of elucidation of the role of *n*-butyl side chain of FA in relation to the inhibitory action, the detailed comparative studies on the types and on the intensities of the inhibitory actions of FA and PA*, which forms the inner metal complex salt of the same intermolecular chelation formula with that of FA, have been made by means of the statistical investigation of n values and ϕ values, calculated by the regression equation. The experimental data for each hindrance of the respiration and the Fe-porphyrin oxidase powers of the test rice seedling were treated by the method of analysis of covariance to establish the regression equation. In each case the regression equation was established repeatedly 4 times. The data for the investigation of n values and ϕ values of each experiment are shown in Tables XII, XIII and XIV. From these data it is revealed that in each experiment between the n values of FA and PA, namely between the types of the inhibitory actions of these two inhibitors, no significant difference is observed and this indicates that the mechanisms of the inhibitory actions of these two inhibitors are identical. From this result an important conclusion has been deduced that *n*-butyl side chain of FA should never directly play a role of the toxic factor by itself. This conclusion is further supported by the finding that FA is completely bereft of its inhibitory action on the catalase activity by the esterification of its carboxyl group (see Table XI). 8HQ, which not only forms a chelate ring catching metal atom but also possesses a phenolic OH as a toxic factor, shows an evidently different type of inhibitory action as compared with FA or PA. The type of the inhibitory action of $CuSO_4$ is quite different from those of the other inhibitors, as the mechanism of the inhibitory action is so.

On the other hand between the ϕ values of FA and PA a significant difference is observed, namely the ϕ value of FA is smaller than that of PA. This finding indicates that in regard to the intensity of the inhibitory action FA surpasses PA. For the reason of this fact the authors intend to offer an interpretation based on the electron theoletical investigation of the chemical structure of FA that "-Inductive Effect" of *n*-butyl side chain of FA induces negative charges on adjacent C atoms and consequently increases the electron density on N atom in the pyridine nucleus, according to the principle that the N atom in pyridine nucleus pulls electrons from the C atoms of α- and γ-position (Diagram 3). If the interpretation is appropriate, in the molecule of FA the "-Inductive Effect" of the *n*-butyl side chain should increase the chelation intensity, strengthening the coordinate binding N→Metal in the chelate ring, and

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* Recently in our laboratory PA has been isolated from the culture liquids of *Piricularia Oryzae Cavara*, the causative mould of the Blast Disease, local name "Imochi Disease", which is the most destructive of the diseases of rice plants.

TABLE XII

Investigations on the Types and on the Intensities of the Inhibitory Actions of Fusarinic Acid, α-Picolinic Acid and $CuSO_4$ on the Respiration of rice Seedlings

Cultivation time	Inhibitor	Average of n values and confidence limits of population mean. Sample size=4 $\alpha=0.05$	Average of ϕ values and confidence limits of population mean. Sample size=4 $\alpha=0.05$
4 *days*	FA	0.877±0.073	0.000746±0.000049
	PA	0.997±0.085	0.00105 ±0.000059
	$CuSO_4$	0.462±0.018	0.000244±0.000011
5 *days*	FA	0.884±0.023	0.000835±0.000063
	PA	0.901±0.037	0.00120 ±0.00011
	$CuSO_4$	0.379±0.013	0.000210±0.000016

α: Level of significance. n: Inhibition type of the inhibitor. ϕ: Molality of the inhibitor for 50 per cent inhibition.

The condition of cultivation of the rice grains and the estimation of the respiration value are the same as in Table II.

The n value and the ϕ value were calculated by the regression equation: $H=n\text{Log}G-n\text{Log}\phi+0.5$

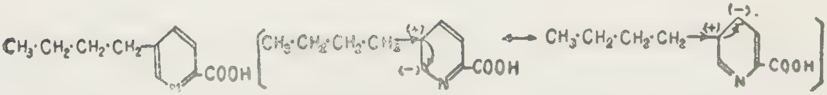

Diagram 3. "—Inductive Effect" of *n*-butyl side chain of FA.

eventually intensify the growth-inhibitory action on plants. This view has been supported by the experimental evidence as shown in Table XV, which demonstrates that the chelation intensity of FA surpasses that of PA. From the above mentioned elucidation it can be concluded that the inhibitory action of FA is due to the chelation mechanism and the *n*-butyl side chain of this acid contributes to the chelation intensity, exercising "—Inductive Effect." The opposite interpretation would be applied to QA which has no remarkable growth-inhibitory action on plants (Photo. 3). In this case "+Electromeric Effect" of the carboxyl group of β-position in the pyridine nucleus induces positive charges on

TABLE XIII

Investigations on the Types and on the Intensities of the Inhibitory Actions of Fusarinic Acid, α-Picolinic Acid, 8-Hydroxyquinoline and $CuSO_4$ on the Catalase Power in Rice Seedlings

Cultivation time	Inhibitor	Average of *n* values and confidence limits of population mean. Sample size=4 $\alpha=0.05$	Average of ϕ values and confidence limits of population mean. Sample size=4 $\alpha=0.05$
4 *days*	FA	1.636 ± 0.141	0.001035 ± 0.000041
	PA	1.625 ± 0.137	0.001202 ± 0.000031
	8HQ	1.237 ± 0.101	0.000690 ± 0.000028
	$CuSO_4$	1.094 ± 0.040	0.000997 ± 0.000021
5 *days*	FA	1.666 ± 0.121	0.000950 ± 0.000040
	PA	1.681 ± 0.115	0.001122 ± 0.000046
	8HQ	1.187 ± 0.043	0.000702 ± 0.000038
	$CuSO_4$	1.076 ± 0.046	0.000969 ± 0.000031

α: Level of significance. n: Inhibition type of the inhibitor. ϕ: Molality of the inhibitor for 50 per cent inhibition.

The condition of cultivation of the rice grains and the estimation of the catalase power are the same as in Table V.

The *n* value and the ϕ value were calculated by the regression equation: $\mathrm{Log}\ H/(1-H)=n\mathrm{Log}G-n\mathrm{Log}\ \phi$.

adjacent C atoms and weakens the chelation intensity of the inner metal complex salt of QA, hindering the resonance effect of the chelate ring (Diagram 4).

Mechanism of the Growth-inhibitory Action of PCA on Plants. The authors further confirmed in the same way as above mentioned that the growth-inhibitory action of PCA on plants was also to be caused by the same chelation mechanism as that elucidated with FA. In this case the methyl group of PCA also contributes to the chelation intensity of PCA, consequently PCA surpassing iso-nicotinic acid in regard to the chelation intensity (Table XV). From the Table XVI it is obvious that between the types (*n* value) of the inhibitory actions of PCA and FA no significant difference is observed, this indicates that the mechanisms of the inhibitory actions of these two inhibitors are identical, and then in regard to the intensity of inhibitory action, which is reciprocal to ϕ value, FA surpasses PCA in proportional to their chelation

TABLE XIV

Investigations on the Types and on the Intensities of the Inhibitory Actions of Fusarinic Acid, α-Picolinic Acid, 8-Hydroxyquinoline and $CuSO_4$ on the Peroxidase Power in Rice Seedlings

Cultivation time *days*	Inhibitor	Average of n values and confidence limits of population mean. Sample size=4 $\alpha=0.05$	Average of ϕ values and confidence limits of population mean. Sample size=4 $\alpha=0.05$
4	FA	1.628 ± 0.051	0.000976 ± 0.000015
	PA	1.630 ± 0.055	0.001049 ± 0.000032
	8HQ	1.184 ± 0.030	0.000627 ± 0.000042
	$CuSO_4$	1.111 ± 0.018	0.000801 ± 0.000052
5	FA	1.665 ± 0.059	0.000843 ± 0.000018
	PA	1.667 ± 0.048	0.000989 ± 0.000046
	8HQ	1.237 ± 0.027	0.000601 ± 0.000033
	$CuSO_4$	1.139 ± 0.024	0.000774 ± 0.000023

α: Level of significance. n: Inhibition type of the inhibitor. ϕ: Molality of the inhibitor for 50 per cent inhibition.

The condition of cultivation of the rice grain and the estimation of the peroxidase power are the same as in Table VI.

The n value and ϕ value were calculated by the regression equation:

$$\mathrm{Log}\ H/(1-H) = n\mathrm{Log}G - n\mathrm{Log}\phi.$$

TABLE XV

Chelation Intensities of the Ferric- and the Copper Complex Salts of Fusarinic Acid, α-Picolinic Acid, etc.

	8HQ	FA	PA	PCA	Iso-NA	FAE	NA
Chelation intensities of the substances with ferric ion (pH 4.0)	0.93	0.66	0.50	0.27	0.24	0.23	0.06
Chelation intensities of the substances with cupric ion (pH 8.0)	0.82	0.54	0.39	0.18	0.15	0.15	0.06

Iso-NA: iso-nicotinic acid FAE: fusarinic acid ethylester
NA : nicotinic acid

intensities (Table XV). As shown in Table XVII and Photo. 5 the injurious effect of PCA on rice seedlings is considerably eliminated by

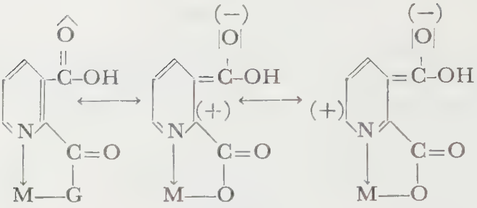

Diagram 4.

" + Electromelic Effect " of β-carboxyl group in the molecule of QA. M : metal

TABLE XVI

Investigations on the Types and on the Intensities of the Inhibitory Actions of α-Picoline-γ-Carboxylic Acid and Fusarinic Acid on the Respiration and on the Fe-porphyrin Oxidase System of the Rice Seedlings

(The investigations have been accomplished respectively in the same way as in Tables XII, XIII and XIV)

Inhibitor	Average of n values and confidence limits of population mean. Sample size=4 $\alpha=0.05$	Average of ϕ values and confidence limits of population mean. Sample size=4 $\alpha=0.05$
	Hindrance of the Respiration	
PCA	0.612±0.028	0.001098±0.000075
FA	0.645±0.032	0.000755±0.000041
	Hindrance of the Catalase Power	
PCA	0.947±0.031	0.00177±0.00014
FA	0.965±0.028	0.00100±0.00007
	Hindrance of the Peroxidase Power	
PCA	1.112±0.038	0.001603±0.00011
FA	1.114±0.033	0.000902±0.00012

α: Level of significans. n: Inhibition type of the inhibitor. ϕ: Molality of the inhibitor for 50 per cent inhibition.

Test rice grains were cultured for 5 days at 30° under the effect of PCA or FA and submitted to each estimation of respiration value, catalase power and peroxidase power. In each case the regression equation was established repeatedly 4 times.

the addition of CaO and then almost completely by the addition of $Fe_2(SO_4)_3$ together with a slight amount of $CuSO_4$, $MnSO_4$ and CaO. It has also been confirmed that PCA is completely deprived of its inhibitory action on the catalase activity by the esterification of its carboxyl group similarly with FA (Table XVIII). PCA was isolated in 1908 by Schreiner and Shorey (*9*) from the soil of a barren land in Takoma district, North America, as a growth inhibitor of plants and they proved that the sterility of the barren land was not only caused by the lack of the nutrition for plants but due to the existence of the growth-inhibitor for plants. In 1916 K. Aso (*10*) also isolated the same substance from the soil of the barren land in Tottori prefecture in Japan.

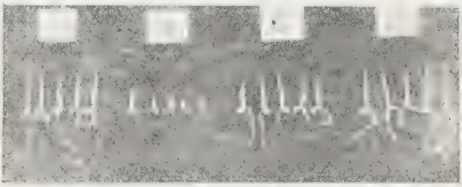

Photo. 5. A: Distilled water B: PCA C: PCA $+Ca^{++}$ D: PCA$+Fe^{+++}+Cu^{++}+Mn^{++}+Ca^{++}$

Test rice grains (Norin No. 1) were cultured with the test solution for 5 days at 30° (See Table XVII)

SUMMARY

By the research on the mechanism of the inhibitory action of fusarinic acid on the plant-growth the following conclusions have been reached.

1. The inhibitory action of fusarinic acid ultimately bases on the chelation mechanism, namely in the plant tissue fusarinic acid hinders the Fe-porphyrin oxidase system catching tightly the heavy metal ions (Fe^{+++}, Cu^{++}, *etc.*), which participate in the formation of this oxidase system, and subsequantly results the check of the plant-respiration.

2. The *n*-butyl side chain of fusarinic acid contributes to the chelation intensity of this acid exercising the "—Inductive Effect" and subsequently to the intensity of the growth-inhibitory action on plants.

3. Further the plant growth-inhibitory action of α-picoline-γ-carboxylic acid, which having once been isolated by Schreiner and Shorey from the soil of barren land in Takoma district, North America, has also been proved to be ultimately based on the chelation mechanism in the same way with that of fusarinic acid.

TABLE XVII

Elimination of the Inhibitory Actions of α-Picoline-γ-Carboxylic Acid on the Root-and Sprout-Growthes, on the Respiration and on the Fe-porphyrin Oxidase Power of Rice Seedlings by the Addition of Metal Ions

(The estimations of the respiration value and the Fe-porphyrin oxidase power of the test rice seedlings have been accomplished in the same way as in Tables II, V and VI)

Test solution	A Distilled water (Control test)	B PCA	C PCA+CA^{++}	D PCA+Fe^{+++}, Cu^{++}, Mn^{++}, Ca^{++}
Average of root-lengths and confidence limits of population mean. *mm.* (α=0.05. Sample size=20)	61.2±6.52	26.3±3.12	48.1±4.31	58.3±5.01
Average of sprout-lengths and confidence limits of population mean. *mm.* (α=0.05. Sample size=20)	50.4±3.15	20.1±1.25	45.5±3.31	50.5±4.12
Average of respiration values and confidence limits of population mean. O_2 *cu.mm.* (α=0.05. Sample size=4)	115.5±3.40	75.3±3.48	108.3±4.72	110.3±5.22
Average of catalase powers and confidence limits of population mean. *N*/20 $KMnO_4$ *ml.* (α=0.05. Sample size=4)	5.41±0.23	3.81±0.21	4.93±0.22	5.15±0.21
Average of peroxidase powers and confidence limits of population mean. guaiacol *mg.* (α=0.05. Sample size=4)	3.30±0.18	2.53±0.14	3.01±0.19	3.15±0.18

α: Level of significans.

Test rice grains (Norin No. 1) were cultured with the test solution (pH 5.5~5.8) for 5 days at 30°.

Test solution B: 0.0028 *M* PCA solution.
,, ,, C: To B an equivalent of CaO was added.
,, ,, D: To B 3/4 equivalents of $Fe_2(SO_4)_3$ and respective 6/1000,000 (weight ratio) of $CuSO_4$, $MnSO_4$ and CaO were added.

Notes: It has been confirmed in advance that metal salts themselves of the above mentioned concentrations have no effects (positive nor negative) on rice seedlings.

Table XVIII

Disappearance of the Inhibitory Effect of α-Picoline-γ-carboxylic Acid on the Catalase Activity by the Esterification of the Carboxyl-group

System	Catalase activity in ml. of $N/50$ $KMnO_4$.
Catalase solution,* 2 ml.+distilled water, 2 ml.	1.36 *ml.* 1.37
,, ,, , 2 ml.+0.05% PCA etylester solution, 2 ml.	1.37 1.38

* Crystalline catalase has been utilized.

This table illustrates that the injurious effect of PCA on the activity of Fe-porphyrin oxidase is due to the function of the carboxyl group, namely to the chelation mechanism as in the case of FA.

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STUDIES ON THE METABOLISM OF THE TUBERCLE BACILLUS WITH THE USE OF RADIOACTIVE SUBSTRATES IN THE PRESENCE AND ABSENCE OF STREPTOMYCIN[1]

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Much of the work done in the search for effective chemotherapeutic agents for tuberculosis has been a blind trial and error testing of numerous chemicals. The number of substances tested to date probably run into several thousands. Out of all this work several good leads have been found, but most of the compounds tested have been found to be either ineffective against the tubercle bacillus or too toxic to animal tissues.

Very little is known concerning the metabolic mechanisms of the tubercle bacillus. This study on the metabolism of a virulent strain of *Mycobacterium tuberculosis* was begun in hopes that the resulting glimmer on the internal workings of this bacteria might serve to guide future searches for effective chemotherapeutic agents. Also, since streptomycin is one of the most effective chemotherapeutic agents against tuberculosis, we thought it would be of interest to study the action of this antibiotic on the metabolism of the tubercle bacillus. In the course of our studies it was shown that streptomycin had no significant effect on the incorporation of radio carbon from acetate and glucose into the lipids of the tubercle bacillus, but had a definite inhibitory effect on the incorporation into the lipid extracted bacterial cell residue (*1*). The present report is on the results of the investigations on the amino acids in the hydrolysates of the lipid extracted bacterial residues.

EXPERIMENTAL

Mycobacterium tuberculosis H37Rv streptomycin sensitive and streptomycin resistant (H37RvSR) variants[2] were used. The organisms were cultivated in Proskauer-Beck liquid synthetic medium for two weeks at 37°. Then approximately one microcurie of the radioactive substrate ($NaHC^{14}O_3$, $CH_3C^{14}OOH$, or C^{14} uniformly labeled glucose) per 200 ml. culture was added, and the cultures were incubated for an additional week, then placed in the deep freeze until a convenient time for processing the contents further. Streptomycin, when added, was added simultaneously with the radioactive substrate, and in a concentration of 100 gamma per ml. of the medium.

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[2] Both strains were kindly supplied by William Steenken, Jr. of the Trudeau Foundation, Trudeau, New York.

The defrosted contents of the flasks were acidified, and thoroughly aerated through a series of NaOH traps to retain any free radioactive carbon dioxide before the flasks were opened, and the bacterial mass, filtered. The bacteria were defatted, dried, and hydrolyzed (*1*, *2*). The amino acids were separated by the chromatographic procedures of Moore and Stein (*3*, *4*), and analyzed by their ninhydrin procedure (*5*) with slight modifications (*2*). Filter paper chromatographies with water saturated phenol were done on all of the amino acids except proline to check on their identities. Proline was readily identifiable by the characteristic yellow color it gave with ninhydrin in contrast to the violet blue of the other amino acid ninhydrin reaction products.

Radioactivity determinations were done with a Technical Associate's GS-4 model scaler attached to a three-chambered gas flow counter tube. Q-gas, a commercial mixture, was used in the tube. All radioactivities obtained were corrected to the theoretical 0 point on a standard self-absorption curve obtained from several determinations at various weights of a standard $CH_3C^{14}OONa$ solution.

In the analyses of the samples from the Dowex 50 chromatograms, it was found that the high content of salt in the effluent added so much weight to the planchets that it interfered with the C^{14} analyses, particularly in samples with very low activities. Therefore, a desalting procedure was devised to remove the excess salts before drying the samples on the planchets for radioactive counts. Short desalting columns of Dowex 50 were made by boring small holes in the bottom of 10×100 mm. test tubes, plugging the holes with washed asbestos fibers, and pouring in the prepared resin to packed levels of about 4 cm. The columns were washed with 1 *N* HCl until acid, then with 10 ml. of 0.01 *N* HCl. Pooled amino acid fractions from the chromatograms were acidified to pH 1 and poured through the columns. The columns were washed with 5 ml. of 0.01 *N* HCl and 10 to 15 ml. of distilled water, and the amino acids were eluted off with several ml. of half saturated ammonium hydroxide solution.

Simple experiments were also conducted to obtain some information on the distribution of the radioactivity in some of the more active amino acids. The ninhydrin reaction which results in the decarboxylation of the amino acids was utilized. Pooled and desalted amino acid fractions from a labeled acetate experiment chromatographed on Dowex 50 were used. The alkaline desalted solutions were aerated to dryness, and the amino acids were redissolved in 5.0 ml. of distilled water. A tenth (0.5 ml.) of this volume was used to determine the total radioactivity of each amino acid. The test tubes with the rest of the solutions were set up for aeration into 0.01 *N* NaOH traps, and 2 ml. portions of the methyl cellosolve-citrate buffer ninhydrin solution were added. The tubes were placed in an aluminum heating block set at about 60°, and slowly aerated for about two hours. Radioactivity determinations were then done on the NaOH solutions and on the ninhydrin reaction residues.

RESULTS

The results are tabulated in the accompanying tables. In the starch chromatograms radioactivity determinations were done on every 0.5 ml. fraction collected, and the specific activities given in Table 1 are the averages of the three to six values obtained for each amino acid peak. The results presented in Tables 2 to 4 inclusive, with few exceptions, are also averages of three or more determinations, while those presented in the rest of the tables on the desalted samples, with few exceptions, are from single determinations.

DISCUSSION

Incorporation of Radioactivity

From Bicarbonate—We began our metabolism studies with labeled bicarbonate. In our preliminary tests we found only very slight incorporation of radioactivity from bicarbonate into the tubercle bacilli even after two weeks' exposure. Fractionation of the bacilli revealed slight activity in the lipids, but none was detected in the amino acids analyzed by starch column chromatography. However, after the development of the Dowex chromatography and the desalting procedure, a single check was made on the bicarbonate experiment with strain H37Rv. The Dowex column allowed the use of larger amounts of material, as well as giving better separation, and the desalting procedure decreased the self-absorption. The improved method resulted in the detection of radioactivity too small to be picked up by the earlier procedure. We were able to detect definite activity in most of the amino acids tested, although the specific activities were uniformly low (Table V). Proline, threonine, and aspartic acid had the highest specific activities, while aspartic acid and glutamic acid had the greatest total activities.

From Acetate—In the acetate experiments the greatest specific activities were found in glutamic acid and in proline in both the sensitive (Tables I, II, and VI) and the resistant (Tables III and VII) organisms. Leucine and aspartic acid also had relatively high activities. Of the three basic amino acids arginine had the highest specific activity (Table X), but it was less than that of glutamic acid or proline. In the starch chromatograms (Table I) the specific activities given for arginine were taken from areas of greatest specific activity. The values at the radioactivity and

TABLE I

Distribution of Radioactivity in Mycobacterium tuberculosis H37Rv
Specific Activities of the Amino Acids

(Starch column chromatograms)

Amino acids	From $CH_3C^{14}OOH$					From C^{14} glucose
Leucine+isoleucine	124	79	65	77	70	37
Phenylalanine	116	82	93	78	55*	121
Valine+methionine	37	34	48	44	40	37
Tyrosine	59*	45*	92*	75*	73*	80*
Proline	210	121	115	127	217	35
Glutamic acid+alanine	83	89	82	95	179	23
Threonine	42	63	40	56	73	19*
Aspartic acid	67	104	89	105	178	27*
Serine	20*	20*	13*	—	15*	0*
Glycine	5*	3*	7*	—	10*	0*
Arginine	112	—	115	—	—	—
Lysine	52*	45*	55*	71*	—	—

* Total counts per minute on planchet were less than 10.

TABLE II

Distribution of Radioactivity from $CH_3C^{14}OOH$ in Mycobacterium tuberculosis H37Rv Specific Activities in Counts/Min./μM

(Dowex 50 chromatograms)

Amino acids	Without streptomycin		With streptomycin		
Leucine	44	55	19*	51	24*
Isoleucine	19*	8*	0*	50*	7*
Phenylalanine	22*	0*	0*	10*	0*
Valine	7*	0*	0*	6*	11*
Methionine	50	6*	19*	61	41*
Tyrosine	37*	0*	10*	26*	0*
Proline	130	89	—	31*	76
Glutamic acid	193	129	—	163	153
Alanine	10*	5*	—	18*	11*
Threonine	38	30	17*	26*	5*
Aspartic acid	50	45	20*	15*	17*
Serine	11*	12*	—	12*	18*
Glycine	8*	12*	—	17*	0*

* Total counts per minute on planchet were less than 10.

TABLE III

Distribution of Radioactivity from $CH_3C^{14}OOH$ in Mycobacterium tuberculosis H37RvSR Specific Activities in Counts/Min./μM

(Dowex 50 chromatograms)

Amino acid	Without streptomycin		With streptomycin
Leucine	137	163	65
Isoleucine	72	86	25*
Phenylalanine	27	34*	10*
Valine	47*	57	0*
Methionine	49	314	0*
Tyrosine	34	24*	20*
Proline	257	247	160
Glutamic acid	169	327	104
Alanine	26	18*	12*
Threonine	55	57	51
Aspartic acid	71	87	37
Serine	24*	25*	10*
Glycine	13*	17	8*

* Total counts per minute on planchet were less than 10.

TABLE IV

Distribution of Radioactivity from C^{14} Glucose Specific Activities in Counts/Min./μM

(Dowex 50 chromatograms)

Amino acids	H37Rv Without streptomycin		H37RvSR Without streptomycin	H37RvSR With streptomycin
Leucine	34	33	20	20*
Isoleucine	31*	0*	15*	14*
Phenylalanine	95*	136	—	108*
Valine	9*	43*	0*	10*
Methionine	0*	61	—	5*
Tyrosine	—	0*	49*	43*
Proline	—	37*	30*	25*
Glutamic acid	12*	22*	11*	17*
Alanine	0*	22*	14*	7*
Threonine	14*	18*	13*	—
Aspartic acid	13*	24*	—	—
Serine	21*	24*	5*	15*
Glycine	0*	10*	—	7*

* Total counts per minuts on planchet were less than 10.

Table V

Distribution of Radioactivity from $NaHC^{14}O_3$ in
Mycobacterium tuberculosis H37Rv

(Desalted samples of Dowex 50 chromatogram)

	On planchet			On column	
	c. per min.	μM of acid	c. per min. per μM	c. per min.	μM of acid
Leucine	7	4.05	2	12	5.97
Isoleucine	26	2.53	10	30	2.97
Phenylalanine	9	0.93	10	17	1.73
Valine	27	4.85	6	31	5.17
Methionine	12	1.52	8	16	1.96
Tyrosine	5	0.65	8	9	1.12
Proline	17	1.02	17	23	1.38
Glutamic acid	75	6.59	11	76	6.93
Alanine	36	10.1	4	42	10.45
Threonine	50	3.48	14	56	3.98
Aspartic acid	87	5.64	15	91	6.07
Serine	24	3.07	8	28	3.48
Glycine	36	5.12	7	38	5.45

* Total counts per minute placed on column were 950.

the ninhydrin peaks (the two did not coincide) were much lower due to the presence of ammonia in this area.

The total uptake of radioactivity from acetate per unit of bacterial protein appears to be generally greater in the resistant strain than in the sensitive. Also the increase in the specific activity of proline in the resistant strain in some of the experiments was out of proportion to the increase in the other amino acids. Leucine also showed a disproportionately high specific activity in some of the studies with the resistant strain. The differences in the total uptake of radioactivity between the two strains may be explicable on the basis of differences in the number of living organisms in the cultures. Unfortunately, a check was not made on this point. However, assuming that the apparent differences are due to actual differences in metabolic rates, one can postulate two possibilities for the greater incorporation: that it is due 1) to a more rapid metabolic rate with consequent greater uptake of the radioactive substrate or 2) to a slower turnover rate with resulting smaller dilution of the incorporated radioactivity. Due to the long experimental period, the second may be the more likely possibility, for if either the tricarboxyilc or the dicarboxylic acid cycle was operative in the tubercle bacillus for the metabolism of acetate, any acetate produced from the reactions of carboxyl labeled acetate will be nonlabeled. Another angle to be considered is that, although the timing of the experiments was kept as constant as possible, it is possible that we did not terminate all the experiments at the same point in the cultural cycle or the metabolic reaction curve. If the two strains of organisms metabolized at different rates, they would not be at the same stage of metabolism at the same time. This is not an unlikely occurrence, for it has been noted that the resistant strain of *M. tuberculosis* A-27 was retarded in growth (*6*), and in the *E. coli* the ability to oxidize the metabolites of the TCA cycle was reported to be considerably reduced in the resistant strains (*7*). This question can be resolved by running a series of experiments terminated after varying experimental intervals with the two organisms, and comparing the results at comparable stages of metabolism.

From Glucose—The uptake of radioactivity from uniformly labeled glucose by the tubercle bacilli was very much lower than that from acetate. It was so low that without desalting the activities in the amino acids were barely detectable. From glucose the highest specific activities were found in phenylalanine and tyrosine in both the sensitive and the resistant organisms (Tables I, IV, VIII, IX, XI). The specific activities of many of the amino acids were not much higher than that obtained in the labeled bicarbonate experiment. No striking difference in the pattern of incorporation was noted between the sensitive and the resistant strains.

Decarboxylation Studies—The ninhydrin reactions of aspartic acid and leucine from the acetate experiment with H37Rv resulted in the loss of practically all of the radioactivities from the molecules (Table XII). The reactions of glutamic acid and proline, however, left more than half of the activities with the residual solutions. The recoveries of radioactivities were not too good, and the low recoveries can probably be attributed partly to the loss of carbon dioxide during the reaction, since only a single NaOH trap was used with each experimental set up. The ninhydrin reaction results in the deamination and decarboxylation of the amino acids. Since the carboxyl groups beta to, as well as alpha to, an amino group are readily removed by ninhydrin, the reaction of aspartic acid results in the loss of both the carboxyl groups and the formation of acetaldehyde. Acetaldehyde has a boiling point of 21°; therefore, it is quite conceivable that under the conditions of the ex-

Table VI

Distribution of Radioactivity from $CH_3C^{14}OOH$ in Mycobacterium tuberculosis H37Rv Specific Activities in Counts/Min./μM

(Desalted samples of Dowex 50 chromatograms)

Amino acids	Without streptomycin		With streptomycin	
Leucine	65	80	66	42
Isoleucine	21	37	15	9
Phenylalanine	17	27	9*	9*
Valine	17	40	8*	5
Methionine	19	30	74	44
Tyrosine	25	35*	13*	13*
Proline	110	214	51	62
Glutamic acid	146	306	166	145
Alanine	6	21	7	6
Threonine	37	64	24	21
Aspartic acid	47	88	27	29
Serine	14	19	14	9
Glycine	7	15	5	4

* Counts per minute on planchet were less than 10.

Table VII

Distribution of Radioactivity from $CH_3C^{14}OOH$ in Mycobacterium tuberculosis H37RvSR Specific Activities in Counts/Min./μM

(Desalted samples of Dowex 50 chromatograms)

Amino acids	Without streptomycin		With streptomycin	
Leucine	88	164	104	71
Isoleucine	74	76	40	53
Phenylalanine	29	28	17	17
Valine	21	48	13	16
Methionine	58	114	29	41
Tyrosine	33	35	25	25
Proline	320	240	225	289
Glutamic acid	286	368	139	169
Alanine	14	17	10	11
Threonine	90	86	57	40
Aspartic acid	82	99	56	55
Serine	23	14	6	2*
Glycine	6	15	10	9

* Total counts per minute on planchet were less than 10.

TABLE VIII

Distribution of Radioactivity from C^{14} Glucose in Mycobacterium tuberculosis H37Rv Specific Activities in Counts/Min./μM

(Desalted samples of Dowex 50 chromatograms)

Amino acids	Without streptomycin		With streptomycin
Leucine	37	30	5
Isoleucine	45	28	7
Phenylalanine	225	112	12
Valine	30	19	10
Methionine	80	13	13
Tyrosine	90	67	8
Proline	21	34	9
Glutamic acid	19	19	12
Alanine	10	13	3
Threonine	12	16	4
Aspartic acid	15	11	5
Serine	22	20	6
Glycine	17	12	2

TABLE IX

Distribution of Radioactivity from C^{14} Glucose in Mycobacterium tuberculosis H37Rv Specific Activities in Counts/Min./μM

(Desalted samples of Dowex 50 chromatograms)

Amino acids	Without streptomycin		With streptomycin
Leucine	30	32	37
Isoleucine	24	27	29
Phenylalanine	130	154	141
Valine	31	28	16
Methionine	40	17	28
Tyrosine	54	50	64
Proline	29	35	48
Glutamic acid	22	22	21
Alanine	14	12	11
Threonine	15	12	12
Aspartic acid	18	13	13
Serine	21	14	18
Glycine	9	8	5

Table X

Distribution of Radioactivity from $CH_3C^{14}OOH$
Specific Activities in Counts/Min./μM
(Desalted samples of Dowex 50 chromatograms)

Amino acids	H37Rv Without streptomycin	H37Rv With streptomycin	H37RvSR Without streptomycin	H37RvSR With streptomycin
Arginine	62	8	102	83
Lysine	50	9	61	24
Histidine	45	2*	52	20

* Counts per minute on planchet were less than 10.

Table XI

Distribution of Radioactivity from C^{14} Glucose
Specific Activities in Counts/Min./μM
(Desalted samples of Dowex 50 chromatograms)

Amino acids	H37Rv Without streptomycin	H37Rv With streptomycin	H37RvSR Without streptomycin	H37RvSR With streptomycin
Arginine	15	8	12	8
Lysine	6	7	10	16
Histidine	25	6	25	32

Table XII

Partial Degradation Studies on four of the Radioactive Amino Acids
from M. tuberculosis H37Rv Cells Exposed to $CH_3C^{14}OOH$

Amino acids	Radioactivity in counts per minute and per cent of total Total activity	NaOH trap for CO_2		Ninhydrin residue		Total recovered	
			%		%		%
Glutamic acid	1980	495	25	1163	59	1658	84
Aspartic acid	360	255	71	3	1	258	72
Leucine	560	510	91	20	4	530	95
Proline	350	76	22	237	68	313	90

periment it was aerated over with the carbon dioxide. It is also probable that it was not retained in the NaOH trap, especially after drying of the solution on the planchet. Under the circumstances it is not surprising that practically no radioactivity was retained in the ninhydrin reaction mixture, and the low recovery of radioactivity in the aspartic acid experiment may mean that part of the activity was lost with the acetaldehyde portion of the molecule. In the leucine reaction the result is more simply analyzed. Since the removal of the only carboxyl group present removed practically all of the radioactivity, it is clear that practically all of the activity of leucine was in the carboxyl group. The isovaleraldehyde resulting from the leucine reaction has a boiling point of 92.5° which is well above the temperature used in developing the ninhydrin reaction. Glutamic acid, unlike aspartic acid, loses only its primary carboxyl group in reacting with ninhydrin. Since glutamic acid and proline lost only part of their radioactivities on decarboxylation, there is no question that the labeled carbon from acetate was incorporated into other position of these amino acids as well as into the alpha carboxyls.

Metabolic Pathways—There are two pathways by which the radioactive carbon from acetate and glucose may enter the amino acids of the bacterial protein: either through prior complete oxidation to carbon dioxide and fixation of the carbon dioxide, or through an activated two or three carbon fragment by condensation of the fragment with another compound or itself to form a metabolite of a cyclic pool. Approximately 30 per cent of the radioactivity from acetate and about 5 per cent from glucose were recovered as carbon dioxide (*8*). The study with labeled bicarbonate indicates that although some fixation of carbon dioxide occurred in the tubercle bacillus, it cannot account for all the radioactive uptake from acetate and from glucose. Thus, whatever the exact steps are, the second mechanism must be operative in the tubercle bacillus. The evidences concerning the possibility of the occurrence of a tricarboxylic acid or a similar metabolic cycle in the mycobacteria have been excellently reviewed by Edson (*9*).

By the conventional tricarboxylic or the dicarboxylic acid cycle it is possible to obtain labeling only in the carboxyl groups of aspartic acid from carboxyl labeled acetate. This would tend to rule out the possibility that the low recovery in the aspartic acid decarboxylation study was due to loss of activity with the two central carbons. Labeling in glutamic acid from carboxyl labeled acetate can occur also only in the carboxyl groups through the operation of the citric acid cycle. Assuming the biological asymmetry of citric acid on the " three point substrate-enzyme contact " theory (*10*) and the retention of the acetate carboxyl through the cycle, one will obtain, during the first cycling, the labeling in the gamma carboxyl of α-ketoglutarate and from it, gamma carboxyl labeled glutamic acid and ring carbon labeled proline. The labeled α-ketoglutarate will also produce carboxyl labeled oxalacetate which on recycling reactions in the presence of the labeled acetate will form α-ketoglutarate labeled in both the carboxyl groups. This will result in the formation of glutamic acid with labeling in both carboxyl groups and proline with labeling in the carboxyl and a ring carbon. It is also possible for non-labeled acetate formed through the cycle to react with the labeled oxalacetate and eventually produce glutamic acid and proline labeled only in the alpha carboxyl groups. The results obtained on the decarboxylation studies can be explained as being due to mixtures of singly and doubly labeled amino acids.

The metabolism of uniformly labeled glucose through the Embden-Meyerhof scheme would result in the formation of uniformly labeled pyruvate, and from pyruvate, acetate. Evidences are present indicating the occurrence of this reaction scheme in the mycobacteria (*9*), but judging from the very low incorporation of radioactive glucose into the bacilli, it does not appear to play a very prominent role in the metabolic reactions of the tubercle bacillus under our conditions of study. It would be of interest here to study the incorporation of uniformly labeled pyruvate and acetate for comparison.

It is clear that a great deal of work needs yet to be done to clarify the metabolic pathways in the tubercle bacillus. Of particular importance to this specific research will be the more exact and detailed studies on the locations of the radioactivities in the amino acid molecules.

Effect of Streptomycin

The effect of streptomycin on the uptake of radioactivity from carboxyl labeled acetate by the sensitive tubercle bacillus is not a clear cut all or none action. The uptake into the amino acids, glycine, alanine, and serine, which have low activities in the absence of streptomycin, was not particularly affected by the antibiotic. It is unfortunate that a study was not done on the effect of streptomycin on the uptake of radioactivity from bicarbonate. If streptomycin had no effect on the low incorporation of activity from bicarbonate, a possible explanation could be that these amino acids obtained their activities from acetate *via* carbon dioxide, and thus were unaffected. The specific activity of glutamic acid also seems to be unaffected, while uptake into proline, threonine, aspartic acid, and the three basic amino acids showed the most clear cut inhibition. Incorporation into all the others except possibly leucine and methionine, also appears to be inhibited. The specific activity of methionine was somewhat higher in the presence of streptomycin; however, due to the erratic data obtained for methionine on some of the experiments no significance can be attached to this.

The specific activities of the amino acids in the resistant strain exposed to labeled acetate in the presence of streptomycin were also somewhat lower than in the absence of the antibiotic. The decrease here seems to affect practically all of the amino acids, including glutamic acid and leucine, and may be due to a dilution factor involving the number of organisms present. Although the specific activity was lower, the total incorporation of radioactivity into the defatted bacterial residue per culture was, if anything, somewhat higher in the presence of streptomycin (*8*).

The effect of streptomycin on the uptake of radioactivity from glucose was more clear cut than the results with the acetate incorporation studies. In the sensitive strain the uptake in the presence of streptomycin into all the amino acids was no more than that found with bicarbonate, a definite decrease for most of the amino acids in comparison to results in the absence of the antibiotic. In the resistant strain streptomycin appears to have no particular effect on the incorporation of radioactivity into the amino acids.

In our experiments streptomycin was added simultaneously with the radioactive substrate. The possibility exists that, had the radioactive acetate been added later, after streptomycin had time to become fully effective, the inhibition of incorporation of radioactivity into the amino acids may have been more distinct. The more clear cut results obtained with glucose may have been due to a slower utilization of this substrate by the tubercle bacillus. Another factor which may have contributed to the ambiguity of some of our data is the difference in the amount of growth from one experimental flask to another. We were using a virulent strain of tubercle bacillus, and in order to avoid handling the bacilli as much as possible the radioactive substrate and streptomycin were simply added to the grown cultures of the bacillus. One series of experiment was conducted in which the bacilli from several flasks were filtered, and the 4 or 5 g. of bacilli obtained were weighed and carefully divided into equal portions to run in duplicate a study of the incorporation of radioactivity from acetate, with and without streptomycin, using both the H37Rv and the H37RvSR. The bacilli were resuspended in phosphate buffer, and the duration of the experiment was approximately 48 hours. As far as technique went this method was far superior to the one used for most of our experiments, for it permitted the control of the amount of organisms present in each experimental flask, and by portioning out the bacteria from a common pool for each stain, we tended to eliminate any differences in the percentage of living bacteria from flask to flask of the same strain. The use of phosphate buffer also eliminated the complications of the possible effect of the medium. However, the uptake of radioactivity under these conditions was very small, only about 30 counts per minute per mg. of defatted bacteria in the sensitive strain in the absence of streptomycin and 2 counts per minute per mg. in the presence of streptomycin. About 95 per cent of the radioactivity was recovered in the phosphate buffer (*8*). Further experimentations are needed to work out the best possible conditions for radioactive substrate incorporation studies with the tubercle bacilli.

SUMMARY

The incorporation of radioactivities from $CH_3C^{14}OOH$ and C^{14} uniformly labeled glucose, in the absence and presence of streptomycin, by *Mycobacterium tuberculosis* H37Rv, streptomycin sensitive and streptomycin resistant (H37RvSR) variants, was studied. A study was also made on the uptake of radioactivity from $NaHC^{14}O_3$ by the sensitive organism.

The incorporation of radioactivity from bicarbonate was uniformly low in all the amino acids. From acetate the highest specific activities were in glutamic acid and in proline, while in the glucose studies phenylalanine and tyrosine had the highest specific activities. The uptake of radioactivity from acetate into the amino acids was somewhat greater in the resistant strain than in the sensitive. Ninhydrin reactions of aspartic acid and leucine removed practically all the radioactivities incorporated from acetate, while in glutamic acid and proline, the reactions left more than half of the activities in the residual molecules.

Streptomycin caused a decrease in the uptake of radioactivity from acetate into most of the amino acids in the sensitive strain. One notable exception was glutamic acid which does not appear to be affected. In the resistant strain some decrease was also noted in uptake of radioactivity from acetate into the amino acids including glutamic acid. Incorporation of radioactivity from glucose into the amino acids in the

sensitive strain was definitely decreased to a level no more than that obtained with bicarbonate by the antibiotic, while incorporation into the resistant strain was apparently unaffected.

The possible explanations and significance of the results observed are discussed.

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NONYLPYRAZOLE, A NEW ANTIMICROBIAL SUBSTANCE

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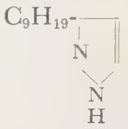
Recently it has been shown by Isogai (*1*) that *Houttuynia cordata* (Dokudami in Japanese), a perenial herb belonging to the saururaceae, contains an aldehydic substance which inhibits the growth of various microorganisms, especially yeast and moulds. This plant is widely distributed in southeastern and eastern Asia and has long been used medicinally by native people for various injuries and diseases such as insect bite, eye-troubles, hemorrhoids, carbuncle, impetigo and other diseases. It seems highly probable that the therapeutic effect of this plant is due, at least partly, to the substance obtained by Isogai.

The chemical nature of the effective substance was studied by one of the present authors (*2*) and was found to be decanoylacetaldehyde: $CH_3(CH_2)_8CO/CH_2 \cdot CHO$, which can be synthesized from methylnonylketone and ethylformate. Based on this finding, an extensive search for more effective antimicrobial substances has been made by synthesizing a large number of related compounds. Thus far the strongest activity was found in 3-n-nonylpyrazole:  which forms the subject of this brief report.

In Table I are given the minimum concentrations of the substance which prevent growth of various organisms *in vitro*. As may be seen, especially vulnerable to the substance are *Staphylococcus aureus*, *Bac. subtilis*, *Zygosaccharomyces*, *Trichophyton* and some other molds. The comparative activities of 3-n-nonylpyrazole, undecylenic acid, pentachlorophenol, sulfathiazole and penicillin against *Trichophyton interidigitale* and

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* Tsuchitoriba-naga-machi, Kanazawa, Japan.

** Mukaino-machi, Takaoka, Japan.

TABLE I

Effect of 3-n-Nonylpyrazole upon the Growth of Various Microorganisms

	Minimum concentration in γ/ml. causing total growth inhibition
Trichophyton interdigitale	2.5
T. purpureum	
Epidermophyton ingguinale	
Trichophyton asteroides	5.0
Aspergillus niger	5–10
Zygosaccharomyces salsus	2.5–5.0
Staphylococcus aureus 209 p	0.5
St. aureus Heatley	
St. aureus Terajima	1.0
Bacillus subtilis	< 1.0
Vibrio cholera	<10
Neisseria gonorrhaeae	10
Streptococcus hemolyticus	1000
Diplococcus pneumoniae	
Escherichia coli communis	>1000
Eberthella typhosa	
Shigella dysenteriae B-III	
Sh. dysenteriae Shiga	
Klebsiella pneumonia	
Pseudomonas aeruginosa	
Proteus vulgaris	

TABLE II

Effect of 3-n-Nonylpyrazole upon Staphylococcus aureus and Trichophyton interdigitale as Compared with That of Some Other Antimicrobial Substances

	Minimum concentration in γ/ml. causing total growth inhibition of	
	S. aureus 209 p	*T. interdigitale*
Nonylpyrazole	0.5	2.5
Pentachlorophenol	0.5	5.0
Undecylenic acid	50	125
Sulfathiazole	>100	
Penicillin	0.05	>100

against *Staphylococcus aureus* are shown in Table II. It is interesting to

TABLE III

Significance of the Length of Alkyl Group for the Antimicrobial Activity of 3-n-Alkyl Pyrazoles

Substituents in 3-n-alkyl pyrazole	Minimum concentration in γ/ml. causing total growth inhibition of		
	Trichophyton interdigitale	*Zygosaccharomyces salsus*	*Staphylococcus aureus* 209 p
Methyl	500	1000	1000
Propyl	125	500	500
Amyl	50	25–50	100
Heptyl	25	10–25	12.5
Octyl	10	5–10	5
Nonyl	2.5	2.5–5	0.5
Undecyl	5	10	1
Pentadecyl	250	500–1000	125

note that in respect to antimicrobial activity, the optimum number of carbon atoms in the alkyl group in 3-n-alkyl pyrazoles is C_9, as is apparent from the data presented in Table III.

The possibility of therapeutic application of the substance is now being studied and will be reported in the near future.

The authors are much indebted to Prof. Dr. H. Tamiya and Prof. Dr. E. Ochiai for their interest and advice during the present study.

EXPERIMENTS

Syntheses of Nonylpyrazole

1) Prepare sodium salt of decanoylacetaldehyde by the condensation of 170 parts of methylnonylketone with 148 parts of ethylformate in 800 parts of ether in the presence of 46 parts of sodium (see Kosuge, 2). Dissolve the product in water, add a mixture of 260 parts of hydrazine sulfate and 80 parts of sodium hydroxide, and leave the solution for about 5 hours. On acidifying the mixture with hydrochloric acid, nonylpyrazole is immediately produced. Purification by means of distillation under diminished pressure yields 140 parts of nonylpyrazole (71 per cent of the theoretical amounts), b.p.$_{22}$ 195°.

2) Dissolve 4 parts of nonyl-β-chlorovinylketone, 1 parts of hydrazine hydrate and 2 parts of potassium acetate in 40 parts of ethanol,

and heat the mixture for 5 hours on a water bath to drive off the ethanol. After neutralizing the solution with sodium hydroxide, extract the upper layer with ether. By distillation of the extract under diminished pressure, 3 parts of nonylpyrazole is obtained (83 per cent of the theoretical amounts).

Antimicrobial Test

Trichophyton interdigitale: 20 mg. of each compound was dissolved in 20 cc. of alcohol, to which Sabouraud's medium was added to make a 1 : 10,000 dilution of the compound. This solution was further diluted serially with the same medium and dispensed in test tubes, to which the milky suspension of micro-organism was inoculated. The tubes were incubated at 27° for 4 days before readings were made.

Staphylococcus aureus: The procedure was virtually the same as above, with the exception that broth was used as medium and the incubation made at 37° for 2 days.

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(*1*) Isogai, H., Scientific Papers, *Coll. of Education Univ. of Tokyo*, **2**, No. 1. (1952)
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THE OXIDATION MECHANISM OF GLYCOLIC AND *l*-LACTIC ACIDS BY THE ENZYME FROM LEAVES*

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Recently it was found by Tolbert *et al.* (*1*, *2*, *3*) that the green parts of higher plants contain a very active enzyme which oxidizes α-hydroxymonocarboxylic acids such as glycolic and lactic acids. Working with partially purified and lyophilized enzyme preparation, these authors believed to have shown that glycolic and lactic acids were oxidized in the following manner:

$$\text{Glycolic acid} \xrightarrow{\frac{1}{2}O_2} \text{glyoxylic acid} \xrightarrow{\frac{1}{2}O_2} \text{formic acid} + CO_2,$$

$$\text{Lactic acid} \xrightarrow{\frac{1}{2}O_2} \text{pyruvic acid} + H_2O.$$

They claimed that during these reactions no hydrogen peroxide was formed.

A critical study of their protocols led us to reinvestigate the matter in detail, and it was found that the reactions in question actually involve the formation of H_2O_2 which is decomposed, partially or completely, by the catalase or peroxidase present in enzyme preparations. Based on these observations the data reported by Tolbert *et al.* were reinvestigated and a new mechanism was proposed for the reactions brought about by the enzyme system. Experiments and data bearing on the subject are presented herewith.

MATERIALS AND METHODS

α-Hydroxymonocarboxylic Acid Oxidase—As the source of the enzyme, leaves of KOMATSUNA (*Brassica campestris*) or spinach were used. The enzyme was prepared according to the method of Tolbert *et al.* with minor modifications; namely, by grind-

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* While this paper was in preparation, a report by Zelitch and Ochoa (*J. Biol. Chem.*, **201**, 707, 719 (1953), announcing the same experimental results as ours became available to us.

ing leaves in coolness, pressing out the juice, which was then adjusted to pH 4.3* and freed from chloroplasts and other fragments by centrifugation, and by subjecting the cytoplasmic juice to Na_2SO_4 and $(NH_4)_2SO_4$-fractionations at about pH 7.4. The precipitated enzyme was contaminated with a considerable amount of catalase, most of which, however, could be removed by acidifying the enzyme solution to pH 4.5 and centrifuging off the precipitates. The enzyme solution thus obtained was almost colorless, but assumed, on adjusting to pH 7.6, a light yellow color and showed a strong green fluorescence upon irradiation with ultraviolet light. This solution contained still a small quantity of catalase (activity ratio$=1\sim2$. Ref. *Section I*), which could not be eliminated by such treatments as reacidification, adsorption by Ca-phosphate, *etc.* without causing a more or less remarkable loss of the enzyme activity. The enzyme preparations obtained by Na_2SO_4- and $(NH_4)_2SO_4$-fractionations containing different amounts of catalase were used in our experiments.

In some experiments the following enzymes were also used:

Catalase—extracted from equine liver and purified by the method of Kitagawa-Shirakawa (*4*).

Peroxidase—Prepared from the root of DAIKON (*Raphanus acanthiformis*) by Yakushiji's procedure (*5*).

Carboxylase—As a crude sample of which a suspension of yeast powder was employed.

Measurement of gas exchanges was carried out using Warburg's monometer at 15°, with the reaction mixture of 10 ml. containing borax-phosphate buffer of pH 8.8, 10 μM of glycolic acid and the enzyme. Prior to each experiment the reaction mixture was freed from CO_2 by evacuating the enzyme solution as well as by heating and evacuating the buffer solution. Gas phase of the vessel was filled with air, and no KOH solution in the center well. Because of the high pH value of the reaction mixture, all of the CO_2 produced during the experiments was bound in the solution. At the end of the experiment, the amount of CO_2 produced was determined by tipping 0.5 ml. of H_2SO_4 (1 : 5) into the reaction mixture from the side bulb.

RESULTS

Oxidation of Glycolic Acid

I. *Total Uptake of Oxygen and Output of Carbon Dioxide, and Their Dependency upon the Catalase Content in the Reaction Mixture*—When the enzyme was shaken with glycolic acid in the presence of air, oxygen was taken up with a steady rate at first, but later it gradually tapered off and ceased completely at the point of substrate exhaustion. At this point the amount of CO_2 released was measured by dumping the acid solution into the main chamber of vessel and it was compared with the total amount of oxygen consumed. The striking fact was that the total amounts of O_2 consumed and CO_2 released varied with the catalase content of the enzyme preparation used, which as mentioned already, varied according to the preparative method applied. The catalase content in different enzyme preparations was compared by the ratio of the activities of catalase and the oxidase (activity ratio=relative activity of catalase: relative activity of the oxidase, the latter being determined by measuring the initial rates of O_2-output and -uptake per 5 minutes at the optimum concentrations of substrates: $M/50$ for H_2O_2 and $M/1000$ for glycolic acid). In Table I are compared the total uptake of O_2 and output of CO_2 as they vary according to the relative activity ratio of the two enzymes (see also Fig. 1).

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* Tolbert *et al.* had adopted the pH value of 5.2 which was found to be inadequate in our experiment, because at that pH a considerable amount of chloroplasts containing very active catalase was left in the supernatant after centrifugation. To remove chloroplasts, pH values of 4.3 and 4.6 were found to be suitable for spinach and KOMATSUNA, respectively.

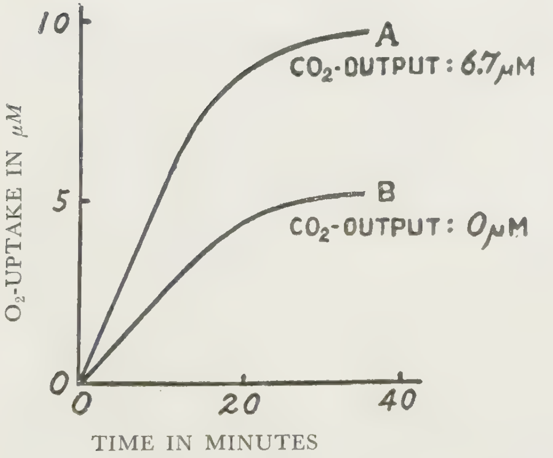
TABLE I

Total Uptake of O_2 and Output of CO_2, and Their Dependency upon the Catalase Content in the Reaction Mixture

Activity ratio	O_2 absorbed	CO_2 released
	μM	μM
2	9.6	7.8
20	7.2	3.8
∞ (a)	5.2	0.1

(10 μM glycolic acid was added)

(a): Concentrated equine liver catalase was added to the reaction mixture.



A: Without the addition of catalase. (activity ratio=*ca.* 2)

B: With catalase added in excess.

FIG. 1. Effect of catalase on the O_2-uptake and CO_2-output.

As may be seen from the table the amount of O_2 absorbed and CO_2 released decreased with the increase of catalase present. The CO_2-output even ceased completely when catalase was added in excess. Fig. 1 shows that not only the total amount of O_2 consumed but also its initial rate was affected by the existence of catalase in excess. The depression of both quantities was about 50 per cent when compared with the results obtained with the enzyme sample showing a very low activity ratio of catalase.

It should be noticed that if 10 μM glycolic acid is oxidized completely to formic acid and CO_2, 10 μM each of O_2-uptake and CO_2-output should occur. The result obtained with the catalase-poor preparation is approximately in accord with this expectation. The fact that, in the presence in excess of catalase, oxygen uptake is reduced to 50 per cent, while CO_2-output ceases completely leads us to suppose that (i) the oxidation of glycolic acid by the enzyme in the absence of catalase is accompanied by the formation of one mole H_2O_2 per mole glycolic acid consumed and that (ii) the accumulation of H_2O_2 in the reaction mixture is a necessary condition for the liberation of CO_2.

II. Effect of Azide, Cyanide and Dimedone—To test the assumption described above, attempt was made to exclude completely the participation of catalase in the course of event by adding sufficient amounts of the well-known catalase inhibitors, cyanide and azide, to the reaction mixture. The data presented in the Table II show that the two inhibitors applied brought about distinctly different results. The effect of azide can be simply accounted for by the blockade of catalase action, whereas cyanide was found to prevent the CO_2-formation, although it causes an O_2-absorption that can be explained by the inhibition of catalase.

The fact that cyanide inhibits the CO_2 liberation leads us to conjecture that during the process cyanide stabilizes a certain intermediary product from which, otherwise, CO_2 is liberated. Since cyanide is known, unlike azide, to combine with carbonyl group, the effect of a typical carbonyl reagent dimedone was investigated.

From Table II it may be seen that in the presence of dimedone no CO_2 was produced, while the O_2-uptake was reduced to a half of the amount observed when azide or cyanide was added to the reaction mixture. These experiments brought out two

TABLE II

Effect of Azide and Cyanide on O_2-uptake and CO_2-output

Conditions	O_2 absorbed	CO_2 released
	μM	μM
Control*	9.4	6.9
+ azide (*M*/1000)	9.6	9.6
+ cyanide (*M*/500)	9.6	0.2
+ dimedone**	5.7	0.07

(10 μM glycolic acid was added)

* Activity ratio = *ca.* 2.

** Saturating concentration in the reaction mixture.

points very clearly: (i) when the catalase activity is inhibited by azide or cyanized, one mole O_2 is taken up per mole of glycolic acid consumed and this is twice the O_2-uptake observed in the presence of sufficient amount of catalase, and (ii) the CO_2-production is inhibited either in the presence of catalase in excess or in the presence of carbonyl reagents such as dimedone or cyanide.

III. Formation of Hydrogen Peroxide—According to the finding described in previous sections, it is clear that H_2O_2 formed during the oxidation of glycolic acid accumulates when both catalase activity is completely inhibited and the intermediary product is stabilized by some carbonyl reagents. The quantity of H_2O_2 accumulated under such a condition was determined by measuring the O_2-evolution caused by the addition of an excessive quantity of catalase at the end of the reaction. From Table III it may be seen that in the presence of both azide and sufficient amount of cyanide, H_2O_2 has been accumulated in approximately equimolar amount as the total O_2 absorbed as well as the glycolic acid consumed, while almost no CO_2 was liberated. In the absence or in the presence of insufficient amount of cyanide, a remarkable decrease in H_2O_2-accumulation and corresponding increase in CO_2-output were observed (2, 3 in Table III). In this case the sum of the amounts of CO_2-output and H_2O_2 accumulated was found to be approximately equimolar to the amount of glycolic acid consumed. It may therefore be inferred that CO_2 is produced by the action of H_2O_2 on some intermediary product on mole to mole basis.

The experiment given on the 5th line of Table III was attempted to make the H_2O_2 accumulated act as an oxidant in the reaction of peroxidase using reduced Bindschedler's green as substrate. In this case both the CO_2-output and H_2O_2-accumulation were negligible in amount, while O_2 was consumed normally equimolecular amount to the glycolic acid disappeared.

The data given on the 6th line of the same table (Table III) were those obtained when the reaction mixture was incubated anaerobically with the addition of 2, 6-dichlorophenol indophenol as a hydrogen acceptor. In this case decolorization of the dye stuff owing to the dehydrogenation of glycolic acid occurred in about 15 minutes, during which time neither H_2O_2 was formed nor CO_2 was produced. It was confirmed that in this reaction one mole of 2, 6-dichlorophenol indophenol has been reduced per mole of glycolic acid added.

IV. Glyoxylic Acid as an Intermediary Product and the Source of CO_2—Based on our inference that CO_2-production in the absence of catalase is caused by the reaction of accumulated H_2O_2 with some intermediary oxidation product, an attempt was made to catch the intermediary product in the reaction mixture in which the accumulation of H_2O_2 was prevented by the addition of a sufficient quantity of pure catalase. After the O_2-uptake proceeded to completion the reaction mixture was deproteinized by three methods: (i) acidification, (ii) addition of trichloroacetic acid, and (iii) boiling at 100°, all of which gave the same results in yielding a solution with which the nature of the intermediate product could be studied.

It was revealed that (i) the intermediary product is an acid and its Ca- and Ba-salts are fairly water-soluble; that (ii) it is not oxidized by glycolic acid oxidase; that

TABLE III

Effect of Several Reagents on H_2O_2-formation

	Conditions	O_2 absorbed	CO_2 released	H_2O_2 accumulated (c)	Consumed substrate as calculated from the amounts of CO_2 & H_2O_2 ($CO_2+H_2O_2$) (d)
		μM	μM	μM	μM
1.	Control	8.7	7.1	0.0	7.1
2.	+azide (*M*/1000)	9.7	8.5	1.2	9.7
3.	+Azide (*M*/1000) (a) +cyanide (*M*/3000)	9.8	5.0	5.0	10.0
4.	+azide (*M*/1000) (a) +cyanide (*M*/1500)	10.5	0.8	9.5	10.3
5.	+reduced Bindschedler's green (b) +peroxidase (concd.)	9.4	1.8	0.0	1.8
6.	+2,6-dichlorophenol indophenol (*M*/500)	(anaerobic)	0.0	0.0	0.0

(10 μM glycolic acid was added in all reaction mixture.)

(a): To suppress catalase action in low concentrations of cyanide, a sufficient amount of azide was always added (see *Section II*).

(b): Reduced Bindschedler's green was proved to be a good hydrogen acceptor for peroxidase.

(c): H_2O_2-formation was measured manometrically by adding concentrated equine liver catalase.

(d): Calculation was made on the assumption that one mole of H_2O_2 was used for the formation of one mole of CO_2.

(iii) on addition of H_2O_2, it spontaneously liberates CO_2 in the amount equivalent to the glycolic acid consumed in the reaction mixture; and that (iv) the reaction between the product and H_2O_2 proceeds faster at higher pH than at lower.

In view of these findings as well as of the data reported by Tolbert *et al.*, one may naturally envisage glyoxylic acid as the intermediary product in question.

Glyoxylic acid was synthesized in three ways: (i) reduction of oxalic acid with Mg, (ii) oxidation of N-dibutyl-tartarate with Pb_3O_4, and (iii) hydrolysis of dibromomalonic acid. When H_2O_2 was added to the solution of the acid, it was, as we have expected, spontaneously decomposed liberating an equivalent amount of CO_2.

V. Oxidation by Plant Sap—Tolbert *et al.* reported in their paper (*3*) that the sap or crude enzyme from older plants took up about one mole of O_2 per mole of glycolic acid, producing only a little CO_2 (RQ=0.1~0.3), and that concurrent with this oxidation there occurred a decolorization of chlorophyll.

We have observed that if the plant sap was treated with H_2O_2 prior to the addition of substrate by dropping gradually 1 *M* H_2O_2 into 10 ml. of concentrated plat

TABLE IV

Spontaneous CO_2-liberation Caused by the Addition of H_2O_2 to the Deproteinized Reaction Mixture

Original reaction mixture contained 50 μM of borax-phosphate buffer, pH 8.8, 1 ml. of glycolic acid oxidase, 0.01 ml. of epuine liver catalase and 10 μM of glycolic acid. Total: 10 ml.

Deproteinizing treatment	CO_2 formed	
	(observed)	(theoretical)
	μM	μM
1) CCl_3COOH-addition* (pH 1.0)	10.1	10.0
2) Boiling at 100°,** (5 min., pH 8.8)	6.8	10.0
3) Acidification*** (pH 1.0)	10.0	10.0

Added H_2O_2: 3% solution, 0.5 ml.

pH at which measurements were made: 3.0 for 1) and 3), 8.8 for 2).

* pH was lowered to ca. 1.0 by CCl_3COOH-addition.

** In this case $M/500$ of NaN_3 was added to suppress the catalase activity.

*** pH was brought below 1.0 with H_2SO_4 (1 : 5).

sap, the evolution of CO_2 was increased to a considerable degree. With the first few drops of H_2O_2, the color of plant sap was changed from green to dark brown, the similar change of color being also observed in the oxidation of glycolic and lactic acids.

TABLE V

Effect of H_2O_2-treatment on the Total Uptake of O_2 and Output of CO_2 by Plant Sap

Condition	O_2 absorbed	CO_2 released	CO_2 released by adding H_2O_2 at pH 3.0
	μM	μM	μM
Control	10.2	0.6	8.1
Treated with H_2O_2	9.8	7.4	—

(10 μM glycolic acid was added.)

Judging from the data given in the last column in Table V, the oxidation product of glycolic acid by plant sap may be the same as that produced by the salt-precipitated enzyme containing catalase in excess.

Oxidation of Lactic Acid

Using lactic acid as substrate, the effect of catalase in modifying the amount of O_2 consumed and CO_2 released was found to be entirely the same as that observed when glycolic acid was used as substrate. Comparison was made of the amounts of pyruvic acid* accumulated in the presence and absence of catalase in excess. Pyruvic acid was assayed manometrically at pH 6.0 by using yeast powder as a crude carboxylase sample. From the data in Table VI it may be concluded that pyruvic acid is not the endproduct but is further decomposed with the liberation of CO_2 when the reaction mixture is poor in catalase.

TABLE VI

Effect of Catalase on O_2-uptake, CO_2-output and the Accumulation of Pyruvic Acid

Conditions	O_2 absorbed	CO_2 released	Pyruvic acid accumulated
	μM	μM	μM
Control	23.0	19.5	8.7
With excess catalase	22.0	0.0	31.0

(250 μM lactic acid was added.)**

DISCUSSION

According to the assumption made by Tolbert *et al.* the oxidation of glycolic acid by the relatively pure enzyme proceeds first through the oxidation by an aerobic dehydrogenation to glyoxylic acid, which then undergoes oxidative decarboxylation to formic acid and CO_2. Results obtained in our experiments, however, revealed clearly that per mole of glycolic acid oxidized one mole each of glyoxylic acid and H_2O_2 was produced, which then reacted with each other, quite non-enzymatically, to produce one mole CO_2.

As the endproduct of overall oxidation of glycolic acid, formation of formic acid was demonstrated by Tolbert *et al.* (*2*), and there seems to be no room left to doubt it. The steps by which glycolic acid is oxidized may, therefore, be written as follows:

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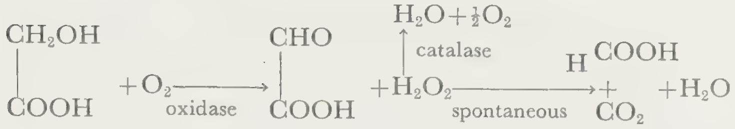
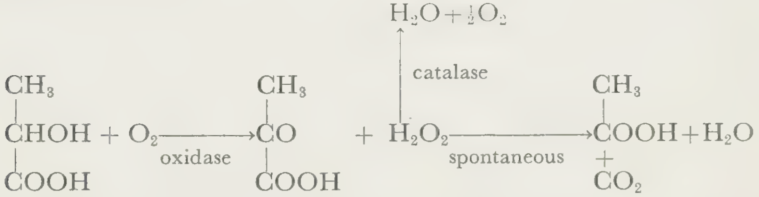
* Tolbert *et al.* reported that pyruvic acid was the main product of lactic acid oxidation by this enzyme.

** The Michaelis constant of lactic acid being very large (about $M/80$) compared with that of glycolic acid, such a large amount of substrate was added in order to make the reaction proceed at an appreciable rate.



$$\begin{matrix}CH_2OH\\|\\COOH\end{matrix} + O_2 \xrightarrow[\text{oxidase}]{} \begin{matrix}CHO\\|\\COOH\end{matrix} + \overset{\overset{H_2O+\frac{1}{2}O_2}{\uparrow\,\text{catalase}}}{H_2O_2} \xrightarrow[\text{spontaneous}]{} \begin{matrix}HCOOH\\+\\CO_2\end{matrix} + H_2O$$

The mechanism of oxidation of lactic acid by the enzyme appears to be essentially similar to that of glycolic acid oxidation. Namely, lactic acid is first oxidized to pyruvic acid with the uptake of one mole of O_2 and production of one mole of H_2O_2 per mole of lactic acid. The H_2O_2 formed will then oxidize the pyruvic acid presumably to acetic acid and CO_2. This assumption seems to be amply justified by the fact that the addition of catalase caused decrease in the rate of O_2-uptake and increase in the quantity of pyruvic acid accumulated, which was assayed enzymatically with carboxylase (See *Section VI*). Acetic acid as the endproduct has not yet been demonstrated, but the formation of any other acid is hardly thinkable. Thus, the probable course of events occurring in the oxidation of lactic acid may be represented as follows:



$$\begin{matrix}CH_3\\|\\CHOH\\|\\COOH\end{matrix} + O_2 \xrightarrow[\text{oxidase}]{} \begin{matrix}CH_3\\|\\CO\\|\\COOH\end{matrix} + \overset{\overset{H_2O+\frac{1}{2}O_2}{\uparrow\,\text{catalase}}}{H_2O_2} \xrightarrow[\text{spontaneous}]{} \begin{matrix}CH_3\\|\\COOH\\+\\CO_2\end{matrix} + H_2O$$

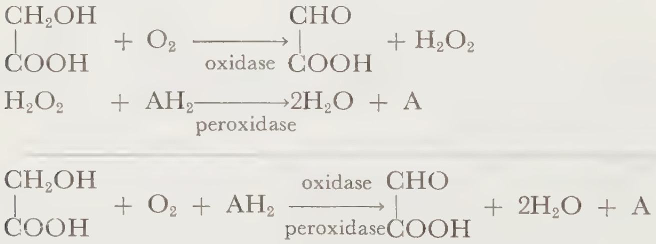
According to Tolbert *et al.*, the oxidation of glycolic acid by the plant sap, showed a small RQ-value, with an O_2-uptake that was equimolar to the glycolic acid consumed. Simultaneously with this oxidation they observed the decolorization of chlorophyll which could be suppressed by the addition of cyanide. These phenomena were interpreted by the authors with the assumption that glycolic acid was first oxidized to glyoxylic acid and then to some substance other than formic acid and CO_2 and presumably some organic peroxide which is responsible for oxidizing chlorophyll.

The observation made with the plant sap by Tolbert *et al.* that the CO_2 evolution was small, while O_2 was taken up in equimolecular amount to the glycolic acid consumed resembles very much the data we have obtained with the salt-precipitated enzyme in the presence of peroxidase and its hydrogen donor. Also in our experiment a color change of plant sap was observed on addition of H_2O_2, indicating that a considerable amount of phenolic substances and presumably of chlorophyll might have been oxidized peroxidatively by the action of plant peroxidase or catalase itself which is known to be able to act peroxidatively under certain conditions. Ascorbic acid which is known to be dealt with by peroxidase was also found to be oxidized when glycolic acid was acted upon by the salt-precipitated enzyme. The fact that the decolorization of chlorophyll during the oxidation of glycolic acid was suppressed by cyanide is interpreted by its inhibitory effect upon peroxidase or catalase.

When the salt-precipitated enzyme of low catalase content was used, the amount of O_2 consumed reached almost its theoretical level but nevertheless, with fairly small CO_2-evolution. This is perhaps attributed to the contamination of peroxidase and its hydrogen donors (See Table I). That in the presence of azide the amounts of CO_2 produced and H_2O_2 accumulated had well accounted for the quantity of glycolic acid consumed seems to be explained by the inhibitory action of azide not only upon catalase but also on peroxidase (See Table III).

To sum up, the oxidation of glycolic and lactic acids by plant sap is supposed to proceed as follows:

Glycolic acid

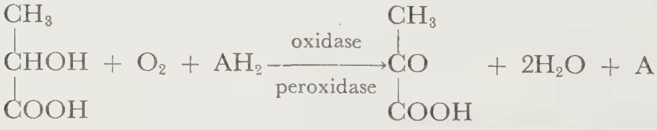


$$\begin{array}{l}CH_2OH\\|\\COOH\end{array} + O_2 \xrightarrow[\text{oxidase}]{} \begin{array}{l}CHO\\|\\COOH\end{array} + H_2O_2$$

$$H_2O_2 + AH_2 \xrightarrow[\text{peroxidase}]{} 2H_2O + A$$

$$\begin{array}{l}CH_2OH\\|\\COOH\end{array} + O_2 + AH_2 \xrightarrow[\text{peroxidase}]{\text{oxidase}} \begin{array}{l}CHO\\|\\COOH\end{array} + 2H_2O + A$$

Lactic acid



$$\begin{array}{l}CH_3\\|\\CHOH\\|\\COOH\end{array} + O_2 + AH_2 \xrightarrow[\text{peroxidase}]{\text{oxidase}} \begin{array}{l}CH_3\\|\\CO\\|\\COOH\end{array} + 2H_2O + A$$

where AH_2 represents some phenolic substances and probably chlorophyll.

Lastly, a word as regards the nature of glycolic acid oxidase the facts that (i) H_2O_2 is produced when substrates are oxidized by the enzyme, (ii) the oxidase is not inhibited by cyanide, and that (iii) the fairly purified enzyme solution gives green fluorescence upon irradiation with ultra-violet light, seem to indicate that the oxidase is very likely a flavoprotein.

SUMMARY

1. The amounts of O_2 consumed and CO_2 evolved in the oxidation of glycolic acid by the salt-precipitated enzyme were found to be dependent upon the amount of catalase contaminated in the enzyme sample. When catalase was practically absent, the amount of O_2 consumed per mole of glycolic acid oxidized was one mole. When excess of catalase was present the O_2-uptake was reduced to 1/2 of this value.

2. The addition of cyanide caused the accumulation of H_2O_2 together with the disappearance of CO_2-formation without changing the amount of O_2-uptake. The addition of azide, on the other hand, caused the increase in CO_2-formation with no H_2O_2-accumulation, but the same O_2-uptake was brought about as in the case of CN-addition.

3. The addition of peroxidase and its hydrogen donor (reduced Bindschedler's green) caused the disappearance of CO_2-formation and of H_2O_2-accumulation without changing the amount of O_2-uptake.

4. H_2O_2 was proved to react spontaneously with the intermediate as well as with synthetic glyoxylic acid to form CO_2. Some additional evidence was aduced in support of the conclusion that glyoxylic acid is the intermediary product in the oxidation of glycolic acid.

5. Glycolic acids is oxidized by plant sap with an equivalent amount of O_2-uptake but only with a little CO_2-formation. When the sap was pretreated with H_2O_2, CO_2-formation was remarkably increased.

6. The formation of H_2O_2 and accumulation of pyruvic acid were confirmed in the oxidation of lactic acid by the same enzyme in the presence of catalase in excess.

7. Based on these observation it was concluded that the oxidation of glycolic and lactic acids proceeds as follows:

In the enzyme solution,

$$\text{Glycolic acid}+O_2 \xrightarrow[\text{oxidase}]{} \text{glyoxylic acid}+H_2O_2$$

$$\xrightarrow[\text{spontaneous}]{} \text{formic acid}+CO_2+H_2O,$$

$$\text{Lactic acid}+O_2 \xrightarrow[\text{oxidase}]{} \text{pyruvic acid}+H_2O_2$$

$$\xrightarrow[\text{spontaneous}]{} \text{acetic acid}+CO_2+H_2O.$$

In plant sap,

$$\text{Glycolic acid}+O_2+AH_2 \xrightarrow[\text{peroxidase}]{\text{oxidase}} \text{glyoxylic acid}+A+2H_2O,$$

$$\text{Lactic acid}+O_2+AH_2 \xrightarrow[\text{peroxidase}]{\text{oxidase}} \text{pyruvic} + A + 2H_2O,$$

where AH_2 represents some unknown, presumably phenolic, substances present in the plant sap.

The authors wish to thank Prof. Dr. Hiroshi Tamiya for his kind advice and interest in this work. The authors are also indebted to Dr. Yasuyuki Ogura for furnishing purified equine liver catalase used in this study, and to Dr. Takeshi Mori for his helpful suggestions in the purification of the enzyme.

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STUDIES ON AEROBIC DECOMPOSITION OF CYSTEINE BY ESCHERICHIA COLI. I

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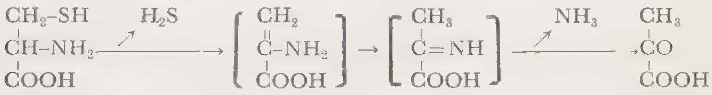
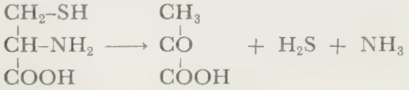
A mumber of organisms are known to produce hydrogen sulfide under certain conditions (*1*, *2*). Recently, Fromageot, (*3*), Binkley (*4*, *5*), Smythe (*6*), Delwiche (*7*), Ichihara (*8*), and Suda (*9*) succeeded in extracting the enzymes from microorganisms as well as from organs of higher animals, which decompose cysteine anaerobically, liberating ammonia and hydrogen sulfide. The majority of these authors admits the reaction mechanism proposed by Fromageot (*10*), who assumed that cysteine is enzymatically desulfhydrated with the formation of α-amino acrylic acid as an intermediate, which should be subject to spontaneous decomposition as follows:



$$\begin{array}{l} CH_2\text{–}SH \\ | \\ CH\text{–}NH_2 \\ | \\ COOH \end{array} \xrightarrow{\nearrow H_2S} \left[\begin{array}{l} CH_2 \\ \| \\ C\text{–}NH_2 \\ | \\ COOH \end{array}\right] \rightarrow \left[\begin{array}{l} CH_3 \\ | \\ C{=}NH \\ | \\ COOH \end{array}\right] \xrightarrow{\nearrow NH_3} \begin{array}{l} CH_3 \\ | \\ CO \\ | \\ COOH \end{array}$$

The overall reaction is:



$$\begin{array}{l} CH_2\text{–}SH \\ | \\ CH\text{–}NH_2 \\ | \\ COOH \end{array} \longrightarrow \begin{array}{l} CH_3 \\ | \\ CO \\ | \\ COOH \end{array} + H_2S + NH_3$$

giving rise to hydrogen sulfide, ammonia and pyruvic acid as the end products.

According to the origin of enzymes or to the conditions of reaction media, intermediary α-amino acrylic acid or pyruvic acid suffers secondary changes and alanine or lactic acid formation is observed (*10*, *6*).

Suda *et al*, suggested, from different point of view, the presence of an unknown phosphorylated compound as intermediate (*9*).

All the authors agree in the view that the decomposition of cysteine occurs anaerobically, although Chaix and Fromageot pointed out that the desulfhydration of cysteine by bacteria was markedly stimulated in the presence of oxygen (*11*, *12*). They reported further that the formation of hydrogen sulfide is accelerated by the addition of glucose (*13*, *14*). However, the reason why oxygen or glucose stimulate the formation of hydrogen sulfide in these cases has not yet been elucidated.

The author (*15*, *16*) of this paper investigated the reaction using *Escherichia coli* as material and was able to show that the decomposition of cysteine with the formation of ammonia and hydrogen sulfide did not take place in ordinary anaerobic condition and that it proceeded rapidly in the presence of oxygen or nitrate together with such substrates as acetate or glucose. The available evidence warranted the conclusion that the mechanism of the reaction observed was essentially different from that assumed for the action of cysteine desulfhydrase upon cysteine under anaerobic conditions. Experiments and data bearing on this subject are presented in this paper.

EXPERIMENTAL PROCEDURES

Cell Preparations—In most experiments, *Escherichia coli*, strain "Taki-2" was used. Similar results were obtained also with other strains of *E. coli* tested, as well as with yeast, *Bacillus subtilis* and *Proteus vulgaris*.

After cultivation for 18 hours at 37°, the cells of *E. coli* were collected and washed three times by centrifugation. In experiments carried out with living cells, suspension of the washed cells in *M*/15 phosphate buffer (pH 6.9) was used. The concentration of the suspension was determined by measuring Kjeldahl's nitrogen. Despite the precautions taken to keep the culture conditions as similar as possible, the specific activity of bacterial cells for cysteine decomposition varied considerably from time to time, the maximal value being almost twice the minimal one.

Very stable cell preparations were obtained by resuspending the washed cells in a very small amount of water, followed by freezing and drying *in vacuo*. The cysteine decomposing activtiy of these preparations was almost equal to that of living cells and remained unchanged for more than several months. The dried preparations were preserved in evacuated desicator in refrigerator.

Conditions of Reaction Media—If not specially mentioned, conditions of reaction media were as follows.

All the components were dissolved in *M*/15 phosphate buffer (pH 6.9). Cysteine hydrochloride was neutralized with Na_2HPO_4 (*M*/15) and then diluted to desired concentration with *M*/15 phosphate buffer, pH 6.9.

The reaction was carried out at 37°, under continuous aeration. This procedure insured not only the sufficient aerobic condition of the medium but also the continuous removal of hydrogen sulfide produced.

Determination of Hydrogen Sulfide—Hydrogen sulfide was determined by the method of St. Lorant (*17*), (*18*). Aerated air was lead in a zinc acetate solution, which captured hydrogen sulfide in the form of zinc sulfide. By the addition of acidic solutions of iron alum and of dimethyl-*p*-phenylene diamine, the sulfur in sulfide was incorporated quantitatively into methylene blue, the blue color of which was measured colorimetrically at 630 mμ. The production of hydrogen sulfide could be traced simply by exchanging the vessels of zinc acetate solution at suitable intervals. Aeration into reaction medium at pH 6.9 was found to be quite sufficient to remove the hydrogen sulfide as soon as it was produced in the medium. This was proved as follows: At the intermediate stage of the reaction, the enzymatic activity was stopped by the addition of concentrated phosphoric acid, then the development of hydrogen sulfide was found to cease also instantaneously, indicating that all of the hydrogen sulfide produced had already been volatilized during the experiment.

Determination of Ammonia—For the determination of ammonia, we used Conway's "unit" (*19*) with a little modification, by which the center dish of it was made removable. The center dish of the unit was filled with 2 ml. of 0.01 *N* sulfuric acid as absorbent for ammonia, and 0.5 ml. of reaction mixture (1–15γ ammonia) and 1 ml. of saturated solution of potassium carbonate were pipetted into the main chamber of the unit. After closing it up tightly,* two solutions in the main chamber were mixed by slanting the unit. After leaving the unit over-night, the acid in the center dish was poured into a test tube using three ml. of distilled water and the amount of ammonia in it was estimated by Russell's colorimetric method (*20*).

Determination of Cysteine—Total amount of cysteine and cysteine was measured by a modified Mecham's method. Two ml. of reaction mixture (5–0.1 micro moles of cysteine) was pipetted into a test tube containing 0.4 ml. of saturated uranyl acetate solution. One ml. of deproteinized supernatant was reduced by zinc sand (60–70 mg.) and hydrochloric acid (4 *N*, 1 ml.), and all cystine was converted into cysteine. After adding ferric salt and dimethyl-*p*-phenylene diamine, the solution was heated in a boiling water bath for 45 minutes. The purple color developed was measured colorimetrically as described by Mecham (*23*).

Measurement of Oxygen Consumption—Oxygen uptake was measured by Warburg's method.

RESULTS

Fundamental Conditions for Aerobic Decomposition of Cysteine by Escherichia coli

Some preliminary experiments were carried out to find the optimal conditions for the reaction. In these experiments only hydrogen sulfide production was followed. In all cases no hydrogen sulfide was pro-

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* We used water-glass (water-glass : water : potassium hydroxide = 2 : 1 : 0.3) as the glue substance for Conway's unit. This agent should be boiled beforehand for 15 minutes.

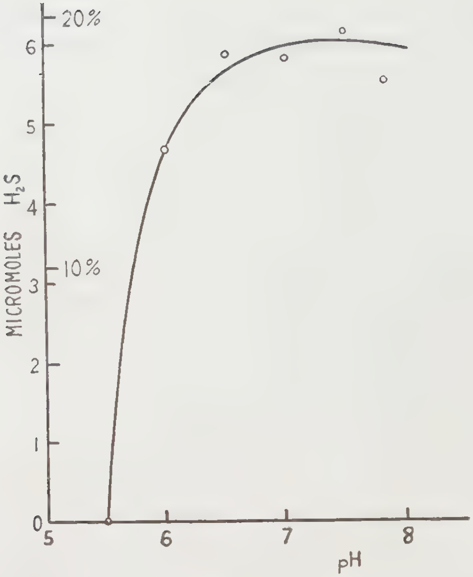

FIG. 1. Effect of pH on cysteine decomposition by *E. coli*. Conditions: Cysteine hydrochloride (31.7 μmoles) and washed cells of *E. coli* (2.4 mg. Kjeldahl nitrogen) were incubated in $M/15$ phosphate buffer (20 ml.) of 37° for 60 minutes under continuous aeration. Gas phase: air.

duced without the addition of cysteine.

Optimum pH—By adding excess of cysteine as substrate, the rates of hydrogen sulfide production at different pH values were compared. As can be seen from Fig. 1, the production of hydrogen sulfide proceeded at an appreciable velocity at pH between 6.5 and 7.5, but it ceased completely at pH 5.5. At higher pH than 7.7, measurement of hydrogen sulfide was impossible by our method.

In accordance with this result, all the following experiments were carried out at pH 6.9.

Dependence of the Reaction Velocity on the Amount of Cells Added—By using cell samples taken from the same culture, it was found that the rate of hydrogen sulfide production was strictly proportional to the amount of the bacteria (Fig. 2). As mentioned already, however, different cultures gave bacteria of different activity.

In the experiment shown in Fig. 3, acetate was added to the reac-

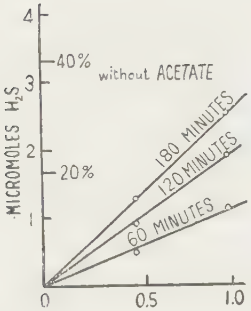

FIG. 2. Influence of the amount of cells on the reaction rate. Contents of each tube: Cystine—8.33 micromoles, washed cells of *E. coli*, *M*/15 phosphate buffer (pH 6.9) to 10 ml. Temperature: 37°. Gas phase: air.

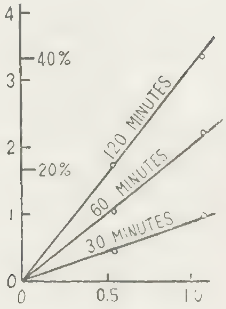

FIG. 3. Influence of the amount of cells on the reaction rate.

Contents of each tube: cystine—8.33 micromoles, washed cells of *E. coli*, acetate—25 micromoles, *M*/15 phosphate buffer to 10 ml. Temperature: 37°. Gas phase: air.

tion mixture, using the same bacterial suspension as that in the experiment of Fig. 2. Comparison of these two figures, shows clearly that acetate markedly accelerates the production of hydrogen sulfide from cysteine under our experimental conditions. Similar effect was observed by many other organic compounds. This problem will be discussed elsewhere in detail. For convenience, these substances, having a promoting effect on the hydrogen sulfide production by bacteria, may be called "promoting substances" in the following.

In these experiments, cystine was used in place of cysteine; under the experimental conditions used, cystine was broken down at almost the same rate as cysteine.

Influence of the Concentration of Substrate on the Reaction Rate—As may be seen from Fig. 4, the initial rate of hydrogen sulfide production from cysteine was independent of the concentration of cysteine at least in the range studied.

It should be remarked that in all cases the rate of hydrogen sulfide production was small at first and increased gradually to show a temporary constant value, after which it tapers off to zero at approximately the time of total consumption of the substrate. Three dashed lines in the figure indicate theoretical values of 100 per cent decomposition respectively.

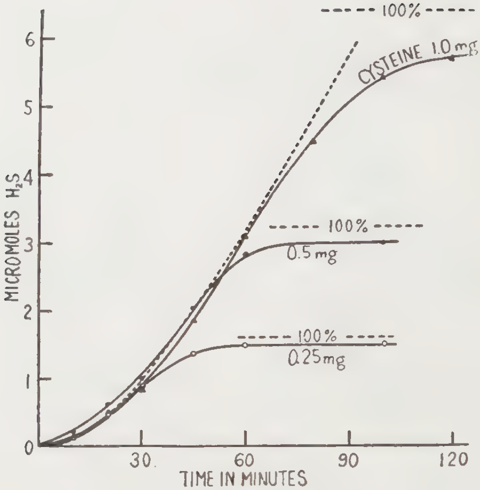

FIG. 4. Effect of substrate concentration on the reaction rate.
Contents of each tube: Cysteine hydrochloride—6.35, 2.17, 1.09 micromoles respectively. Sodium acetate—100 micromoles. Washed cells of *E. coli*—the amount corresponding to 0.83 mg. of Kjeldahl's nitrogen. $M/15$ Phosphate buffer (pH 6.9) to 10 ml. Temperature: 37°. Gas phase: air.

The curves in Fig. 4 may be explained as follows: The process of hydrogen sulfide production observed in our experiment involves at least two rate-limiting reaction steps, of which the one represents a zero order reaction with respect to cysteine concentration, while the other, which is supposed to be the step of hydrogen sulfide production from an intermediate built by the first reaction step, is of first or more order with respect to the intermediate in question. If we assume the second reaction step to be of first order with respect to its substrate, the amount (1) of hydrogen sulfide liberated in the time t should be given by

$$Z = at - \frac{a}{k}(1 - e^{-kt})$$

where a and k are velocity constants of the first and second rate-limiting reactions, respectively. This equation represents the experimental result very well. The dotted curve in Fig. 4 is the theoretical one calculated by the equation, assuming the values of a and k to be 0.965 micromoles/minute and 0.0306/minute, respectively.

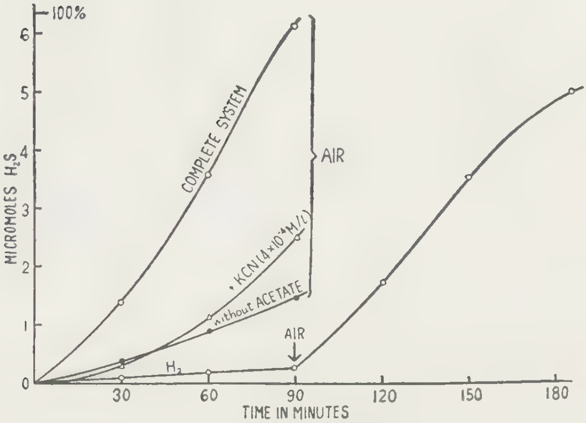

Fig. 5. Oxygen requirement of the reaction.

Contents of each tube: Cysteine hydrochloride—6.35 micromoles. Sodium acetate—50 micromoles. Washed cells of *E. coli*—the amount corresponding to 0.94 mg. of Kjeldahl's nitrogen. *M*/15 Phosphate buffer to 10 ml. (pH 6.9). Temperature: 37°. Gas phase: air or hydrogen as mentioned in the figure.

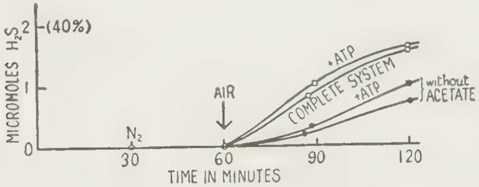

Fig. 6. Oxygen requirement of the cysteine decomposing reaction by *E. coli*.

Contents of each tube: Cysteine hydrochloride—5 micromoles. Glucose—20 micromoles. ATP (Ca salt)—the amount containing 19.3 micromoles of acid labile phosphate. Dried cell preparation of *E. coli*—10 mg. *M*/15 Phosphate buffer (pH 6.9) to 5 ml. Temperature: 37°. Gas phase: nitrogen or air as mentioned in the figure.

Nature of these two reaction steps will be made clearer in later sections.

Requirement of Oxygen or Nitrate for the Cysteine Decomposition by Escherichia coli

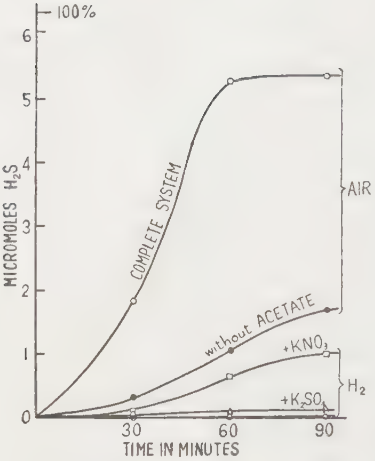

FIG. 7. Effect of nitrate or sulfate on the cysteine decomposition by *E. coli*.

Contents of each tube: Cysteine hydrochloride—6.35 micromoles. Sodium acetate—50 micromoles. Nitrate or sulfate—50 micromoles, when added. Washed cells of *E. coli* which contains 0.94 mg. of Kjeldahl's nitrogen. *M*/15 Phosphate buffer (pH 6.9) to 5 ml. Temperature: 37°. Gas phase: hydrogen or air as mentioned in the figure.

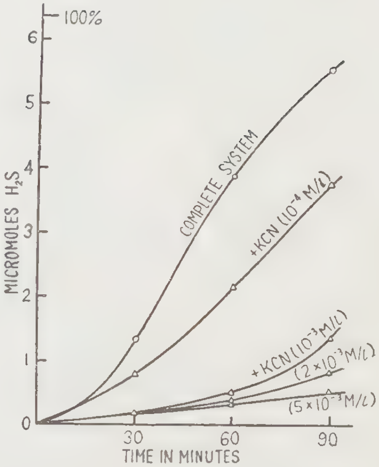

FIG. 8. Inhibition by cyanide.

Contents of each tube: Cysteine hydrochloride—6.35 micromoles. Sodium acetate—50 micromoles. Potassium cyanide. Washed cells of *E. coli* which contains 1.24 mg. of Kjeldahl's nitrogen. *M*/15 Phosphate buffer (pH 6.9) to 10 ml. Temperature: 37°. Gas phase: air.

The decomposition of cysteine by *E. coli* dealt with in this paper is quite different from that of cysteine desulfhydrase in its requirement of specific hydrogen acceptor, oxygen or nitrate.

Effect of Oxygen—In the experiment shown in Fig. 5, hydrogen sulfide production in presence and absence of oxygen was compared. The reaction was remarkably suppressed in hydrogen atmosphere, but when hydrogen was replaced with air (arrow mark in Fig. 5), immediate recovery of the reaction took place. The very small amount of hydrogen sulfide liberated in hydrogen atmosphere is attributable to the contaminating oxygen, since other experiment showed that it was completely halted when oxygen was removed perfectly (see Fig. 7).

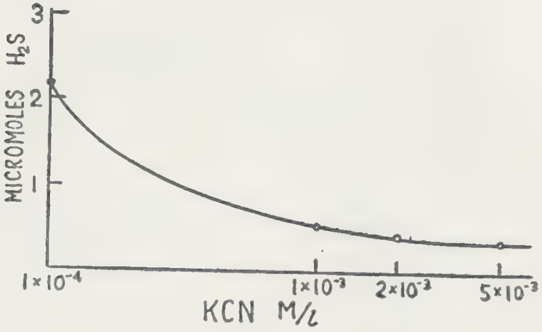

FIG. 9. Inhibition by cyanide.
Conditions are the same as that of Fig. 8.

In the above experiment acetate was used as the promoting substance. Replacement of acetate by glucose and of hydrogen by nitrogen gave the similar result, indicating that the anaerobic breakdown of the sugar has no effect on the liberation of hydrogen sulfide from cysteine by *E. coli* (Fig. 6). Adenosine triphosphate (ATP) was also found to be ineffective as promotor, probably due to its impermeability through cell membrane.

Effect of Nitrate—It is well known that *E. coli* has a strong nitrate reductase. In the cysteine decomposing reaction, nitrate could replace oxygen to some extent, probably by the action of this enzyme. Although to a very small extent, sulfate appeared to have a similar effect in causing an increased production of hydrogen sulfide.

Inhibition by Cyanide—Using acetate as the promoting substance, the effect of cyanide upon the aerobic liberation of hydrogen sulfide was investigated. As shown in Fig. 8, cyanide reduced the rate of reaction even at the concentration of 10^{-4} *M*. The inhibition was, however, incomplete, which was also the case when cyanide concentration was increased hundred times. In the case of lower cyanide concentration, the inhibition decreased with time, which was probably due to the evaporation of hydrogen cyanide during the aeration. Incomplete, but striking inhibitory effect of cyanide was also observed when glucose was used instead of acetate as the promoting substance.

On the available data, we may conclude that for the liberation of hydrogen sulfide from cysteine, the bacterial cells require the existence of oxygen or nitrate which serves as altimate hydrogen acceptors in the

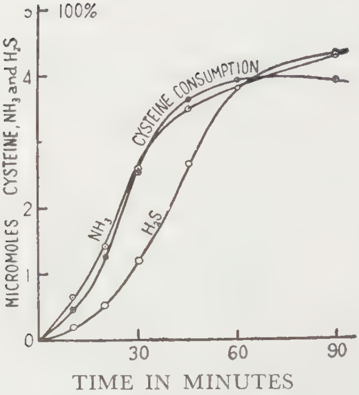

FIG. 10. Interrelation among cysteine disappearance, ammonia production and hydrogen sulfide liberation.

Contents of each tube*: Cysteine hydrochloride—5 micromoles. Glucose—10 micromoles. Dried preparation of *E. coli*—10 mg. $M/15$ Phosphate buffer (pH 6.9) to 5 ml. Temperature: 37°. Gas phase: air.

energy yielding metabolism. The fact that no hydrogen sulfide was produced in nitrogen atmosphere in the presence of glucose indicates that the energy and/or intermediate metabolites produced by the fermentative breakdown of glucose are of no use in provoking the liberation of hydrogen sulfide from cysteine by *E. coli.* It is as yet impossible to say clearly for what reason oxygen or nitrate is necessary for the reaction. It may only be assumed that these oxidants are indispensable either in oxidizing the promoting substance or in oxidizing cysteine itself to produce the conditions necessary for the liberation of hydrogen sulfide.

The inhibitory effect of cyanide on the hydrogen sulfide liberating reaction in air may be taken as an evidence for the functioning of Warburg-Keilin-system in the oxidative phase of the reaction. But the incompleteness of the cyanide inhibition indicates the existence of some effective oxidation mechanism other than Warburg-Keilin system.

The Precedence of the Cleavage of Amino Group of Cysteine to That of Sulfhydryl Group

If the mechanism of aerobic decomposition of cysteine by *E. coli*

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* In the real experiment, four fold amount was used.

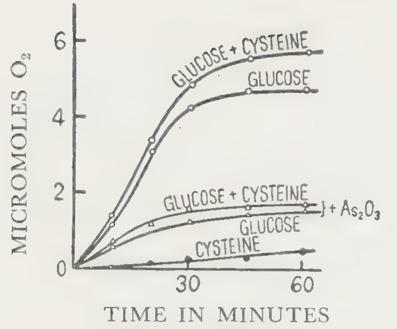


FIG. 11. Inhibition of oxygen uptake by arsenite, with glucose and cysteine as substrates.

Contents of each Warburg vessel: Cysteine hydrochloride—1 micromoles. Glucose—2 micromoles. Arsenous oxide—10 micromoles. Dried preparation of *E. coli*—2 mg. *M*/15 Phosphate buffer (pH 6.9) to 1 ml. 0.2 ml. of 5% potassium hydroxide in center well. Temperature: 37°. Gas phase: air.

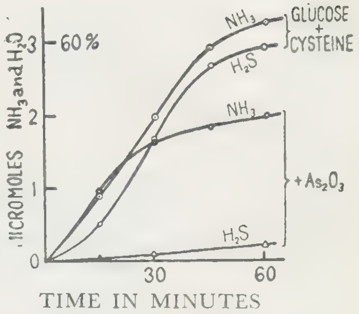


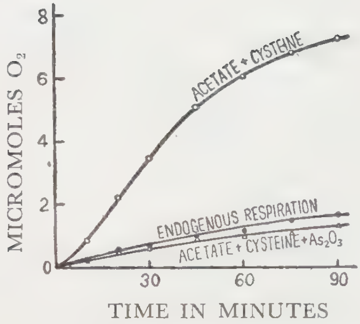
FIG. 12. Effect of arsenite on the cysteine decomposing reaction by *E. coli.*

Contents of each tube: Cysteine hydrochloride—5 micromoles. Glucose—10 micromoles. Arsenous oxide—50 micromoles. Dried cell preparation of *E. coli* —10 mg. *M*/15 Phosphate buffer (pH 6.9) to 5 ml. Temperature: 37°. Gas phase: air.

involves more than two rate-limiting reaction steps, the disappearance of cysteine may probably precede the liberation of hydrogen sulfide. To investigate this point, a series of experiments were performed, using glucose as the promoting substance.

Interrelation among Cysteine Disappearance, Ammonia Production and Hydrogen Sulfide Liberation—In Fig. 10 are reproduced the results of experiments in which not only the decrease in cysteine but also the increase in ammonia and hydrogen sulfide during the reaction were followed. The curve "cysteine disappearance" in Fig. 10 shows the decrease in the total amount of cysteine and cystine. Control value without addition of cysteine was zero. The values for liberated ammonia were those obtained by subtracting from experimental values the amount of ammonia produced in the control experiment in which all components except cysteine were present. All values given are the average of two determinations.

As may be seen from the figure, the disappearance of cysteine coincides almost perfectly with the appearance of ammonia, whereas the



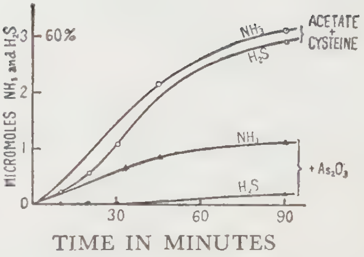


Fig. 13. Inhibition of oxygen uptake by arsenite, with acetate and cysteine as substrates.

Contents of each Warburg vessel: Cysteine hydrochloride—1 micromole. Sodium acetate—5 micromoles. Arsenous oxide—10 micromoles. Dried preparation of *E. coli*—2 mg. *M*/15 Phosphate buffer (pH 6.9) to 1 ml. 0.2 ml. of 5% KOH in center well. Temperature: 37°. Gas phase: air.

Fig. 14. Effect of arsenite on the cysteine decomposition by *E. coli*.

Contents of each tube: Cysteine hydrochloride—5 micromoles. Sodium acetate—25 micromoles. Arsenous oxide—50 micromoles. Dried preparation of *E. coli*—10 mg. *M*/15 Phosphate buffer (pH 6.9) to 5 ml. Temperature: 37°. Gas phase: air.

liberation of hydrogen sulfide remarkably lags behind them. (*9*)

Since the quantity of ammonia and hydrogen sulfide produced amounted to more than 80 per cent of added substrate, it is evident that both ammonia and hydrogen sulfide have been detached from the same molecule of cysteine.

This result, along with the consideration discussed before (see Fig. 4), suggests that the first step reaction coincides with production of ammonia. In other words, the first rate-limiting reaction is, or closely related to, the liberation of ammonia from cysteine. The product of this reaction is then desulfhydrated by the second reaction, which is supposed to be of first or more order with respect to the concentration of intermediate. In the case of Fig. 10, the rate of desulfhydration attained the maximal constant value when the concentration of the intermediate was 1.4 micromoles per 5 ml.

Effect of Arsenite—The fact that the hydrogen sulfide liberation is preceded by the ammonia production was most clearly demonstrated when arsenite was added to the reaction mixture.

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* Values for oxygen uptake given in Fig. 13 are not corrected ones.

Arsenite inhibited the respiration of *E. coli* with glucose and cysteine as substrates. It also inhibited markedly the liberation of hydrogen sulfide from cysteine by the bacteria, but did not affect so much the production of ammonia by deamination of cysteine. (See Figs. 11 and 12)

The values for ammonia given in Fig. 12 are those corrected for the endogenous ammonia production observed in the experiment without added cysteine. The values for oxygen uptake given in Fig. 11 are also those corrected for the endogeneous respiration.

Essentially the same results as above were obtained when acetate was used as the promoting substance (Figs. 13 and 14).

That the marked depression of hydrogen sulfide production by the effect of arsenite was not due to the formation of arsenous sulfide was ascertained by the fact that at the end of the reaction, no hydrogen sulfide was liberated on addition of tin and hydrochloric acid to the reaction mixture, which must liberate hydrogen sulfide, if it contained arsenous sulfide.

Arsenite is known as an inhibitor for malic dehydrogenase which is involved in the tricarboxylic acid cycle. The fact that the ammonia liberation from cysteine is affected by arsenite only slightly, indicates that it may not involve the action of aminophorase system which is known to be operated by the tricarboxylic acid cycle.

Formation of α-Keto Acid in the First Step of Cysteine Decomposition

As the primary deamination product from cysteine, we may naturally envisage thiol-pyruvate. According to Garreau (*22*), this substance is easily attacked by *E. coli* with the liberation of hydrogen sulfide. Moreover, the liberation of hydrogen sulfide from this substance is reported to proceed faster than that from cysteine itself.

In view of the fact that arsenite inhibits hydrogen sulfide liberation more strongly than the deamination of cysteine, an attempt was made to catch the primary deamination product from cysteine by adding arsenite to the reaction mixture.

Two hundred mg. of cysteine hydrochloride, 100 mg. of arsenous oxide and 200 mg. of dried cell preparation of *E. coli* were dissolved in 50 ml. of $M/15$ Na_2HPO_4.* The mixture was aerated at 37° and 40 mg. each of glucose was added at the intervals of 100 minutes. At each sampling, the pH of the reaction mixture was tested and the mixture was kept neutral by adjusting pH with 5 N sodium hydroxide. After 300 minutes of incubation, the reaction mixture was deproteinized by adding 5 g. of trichloracetic acid. Into the centrifuged supernatant, hydrogen sulfide gas was introduced for three hours, after which hydrogen sulfide was driven off by nitrogen gas, and the solution was neutralized using methyl red as indicator. On adding a small amount of saturated solution of cupric sulfate to the solution, precipitates of mercaptane and cupric phosphate was formed, a reaction which is characteristic for sulfhydryl group. The precipitates were separated by centrifugation and dissolved in 5 ml. of 4 *N* hydrochloric acid. To this acidic solution, equal volume of saturated 2,4-dinitrophenyl hydrazine in 4 *N* hydrochloric acid was added. After standing overnight in refrigerator, there was formed a red crystal. The crystal obtained melted at 200–205°, and showed a nitrogen content of 18.44 per cent. 2,4-Dinitrophenyl hydrazone of thiol pyruvate should melt at 195–200° (21) and contain 18.66 per cent nitrogen. This accordance indicates that the primary deamination product we have obtained was probably, as we have expected, thiol pyruvate.

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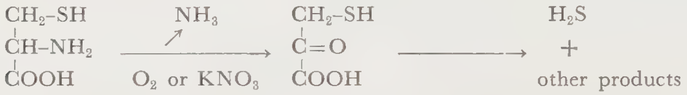
* By this solvent cysteine hydrochloride was neutralized to pH 6.9.

DISCUSSION

From the experimental results described above, the most probable mode of reaction in the decomposition of cysteine by *E. coli* may be pictured as follows:



$$\begin{array}{c} CH_2-SH \\ | \\ CH-NH_2 \\ | \\ COOH \end{array} \xrightarrow[O_2 \text{ or } KNO_3]{NH_3 \nearrow} \begin{array}{c} CH_2-SH \\ | \\ C=O \\ | \\ COOH \end{array} \longrightarrow \begin{array}{c} H_2S \\ + \\ \text{other products} \end{array}$$

This reaction mechanism is essentially different from that assumed for the action of cysteine desulfhydrase, and it is especially featured by the fact that it proceeds only in the presence of oxygen or nitrate and is accelerated remarkably by the addition of respiratory substrates such as sugar or acetate. It will be shown in the forthcoming paper that these respiratory processes are linked with the first step in which cysteine is attacked with the liberation of ammonia.

The decomposition mechanism of thiol pyruvic acid is yet obscure. We only know that thioglycolic acid is not effective as the source of hydrogen sulfide in the bacteria, and therefore it does not appear to be the intermediate in thiol pyruvic acid breakdown.

The author is very grateful to Prof. T. Soda for his continuing guidance through the course of this research. The author also wishes to thank Drs. Y. Kiho, Y. Kondo and J. Niwa for their co-opperations and many useful discussions.

SUMMARY

1. Cysteine was decomposed by living cells as well as by dried cells of *E. coli*. Hydrogen sulfide and ammonia were detected as the

products.

2. The optimum pH of the reaction was about 7.

3. Initial velocity of the reaction was proportional to the amount of cells added.

4. Under the conditions used in the experiments, the rate of hydrogen sulfide production from cysteine by *E. coli* was independent of the cysteine concentration. The curve of hydrogen sulfide liberation was in good agreement with the theoretical one, calculated by assuming a stepwise reaction mechanism.

5. The cysteine decomposing reaction by *E. coli* occurred only in the presence of oxygen or nitrate. The reaction was strongly inhibited by hydrogen cyanide.

6. Liberation of hydrogen sulfide from cysteine was preceded by cysteine disappearance and by detachment of amino group from cysteine.

7. When arsenite was added to the reaction mixture, there occurred ammonia production without liberation of hydrogen sulfide.

8. In the presence of arsenite, a thiol keto acid was obtained from the reaction mixture. This substance was shown to be most probably thiolpyruvic acid.

9. The difference between the reaction and the corresponding reaction brought about by the action of cysteine desulfhydrase was discussed.

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INTERRELATION BETWEEN THE CATALYTIC FUNCTION OF HEME-PROTEINS AND THE STRUCTURAL MODIFICATIONS OF THEIR PROTEIN PARTS

I. SPECTROSCOPICAL OBSERVATIONS MADE ON THE REACTION BETWEEN HEMOGLOBIN AND BENZOATE

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(Received for publication, October 22, 1953)

The physiological function of hemoglobin is maintained, to a large extent, by the native structure of its protein part. Certain structural modifications of the protein part of hemin enzymes may easily entail a profound alteration in their fundamental function. In general, the catalytic function of hematin-Fe can markedly be influenced by coordination of certain basic compounds or proteins, each compound or protein effecting in different ways and grades.

As to the structural modifications of the heme proteins, the work by Anson and Mirsky (*1*, *2*) may firstly be referred. From their spectroscopical observations, they have proved a denaturating effect of salicylate upon the protein part of hemoglobin. Roberts (*3*) studied later the effect of salicylate and benzoate upon methemoglobin and carboxyhemoglobin. It was reported by him that the absorption figure observed in the product of methemoglobin formed by the effect of benzoate or salicylate was markedly similar to that of carboxyhemoglobin. For the reaction of benzoate upon methemoglobin, he calculated the reaction heat to be zero. From both of these findings, he suggested a combination of heme-Fe with salicylate or benzoate, and concluded that the denaturation has possibly occurred in the protein part of the molecule.

The effects of salicylate, benzoate and some other substances upon reduced hemoglobin were further studied by H. F. Holden (*4*). By the addition of these agents in certain concentrations, the absorption figure of hemoglobin was converted into that of hemochrome derivatives. In most cases the reactions were proved to be reversible. He assumed that, in these cases, a slight and reversible denaturation ("perturbation" according to the designation of Holden) has occurred in the protein part.

Holden reported further that the modified hemoglobin thus obtained promoted greatly the reaction of green pigment formation from hemoglobin molecule. In spite of these studies, the mode of reaction of these perturbators upon heme-proteins remains as yet unclarified.

If the effects of these "perturbators" may essentially be taken as one of the denaturation as postulated by Anson, Mirsky and Holden, it may be possible to expect further that the essential function of hemoglobin can be qualitatively altered more or less along with the structural change. From the interest on these points, the present author intended to study more precisely on the absorption change of hemoglobin and methemoglobin occurring under the effect of benzoate.

EXPERIMENTALS

Materials

Reduced Hemoglobin Solution—Bovine erythrocytes were repeatedly washed with physiological saline solution, laked by addition of distilled water and centrifuged. The supernatant of oxyhemoglobin solution was reduced by addition of small amount of powdered $Na_2S_2O_4$.

Methemoglobin Solution—Sodium nitrite was added to the bovine erythrocytes suspension in saline solution in order to carry out the oxidative conversion of hemoglobin to methemoglobin in red cells, so that the excessive nitrite could easily be removed by repeated washing of the cells with saline solution. The washing was repeated more than ten times. The red cells were then laked with distilled water and centrifuged. The supernatant was used as the methemoglobin solution. Methemoglobin prepared from oxyhemoglobin by oxidation with ferricyanide, and subsequently dialyzed against water, gave similar results of experiments as the preparation by nitrite.

Method—For spectroscopic determinations, a spectrophotometer of Hitachi Company was used. The reaction of reduced hemoglobin solution was prepared from oxyhemoglobin solution every time by the addition of small amount of $Na_2S_2O_4$, covered with liquid paraffin and the benzoate solution was then added to the solution. Readings were made after the reaction was completed.

The experiments with methemoglobin and benzoate were carried out in small beakers, except the experiment for the determination of reaction velocity.

The concentrations of hemoglobin was determined, after converting it to alkali-denatured globin hemochrome, by the measurement of the optical density of the latter at 558 $m\mu$. Experiments were carried out at room temperature except otherwise indicated. All the blood was used within 2 days, during which time it was kept in an ice box.

RESULTS AND DISCUSSION

I. Reaction of Methemoglobin with Benzoate

After the addition of benzoate to methemoglobin solution in the final concentration

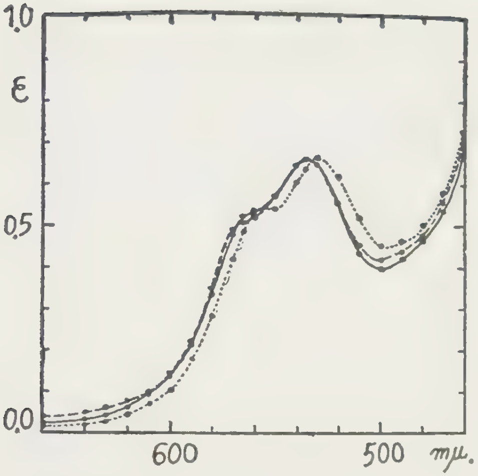


Fig. 1. Absorption figures of hemichromes obtained by addition of benzoate or salicylate and that of pyridine hemichrome.

Full line: obtained by addition of benzoate in its final concentration of 1.0 *M.*

Broken line: obtained by addition of salicylate in its final concentration of 0.5 *M.*

Dotted line: figure of pyridine hemichrome.

of benzoate at 1.0 *M,* an absorption figure was obtained as given in Fig. 1 (full line). The reaction was complete at this benzoate concentration; the resulted absorption figure remained unchanged on further addition of benzoate. For comparison, the absorption figures of pyridine hemichrome and of methemoglobin reacted with 0.5*M.* salicylate are given in Fig. 1. These three compounds showed, by and large, a similar absorption figure. The absorption maximum of pyridine hemichrome was situated, however, at 530 mμ, while that of the others were at 535 mμ.

As shown in Fig. 1, the figure corresponded to that of hemichrome. From this, it will be assumed that methemoglobin structure has possibly been converted into the coordination structure as it is the case in pyridine hemichrome. And if the coordinating atom is N (as in pyridine hemichrome), a globin N should be taken into consideration as the coordinating atom in these new compounds, linking from the other side of their heme-Fe. Wyman *et al.* (*5*, *6*) assumed that the heme-Fe in hemoglobin molecule lies between two imidazole groups of the globin molecule. It may thus be possible to assume that another Fe linking group in the globin molecule has become accessible to coordination to heme-Fe, to form a similar structure as alkali denatured globin hemichrome, along with the slight structural modification of the molecule caused by the addition of salicylate or benzoate.

The reaction between methemoglobin and benzoate were, then, more precisely studied. A series of different absorption figures were obtained by benzoate in varied concentrations in the range below 0.9 *M*, as shown in Fig. 2. Furthermore, two isosbestic points were clearly demonstrated in the figure, indicating a reaction system of two components. Above 0.9*M* of the benzoate concentrations, the final absorption figures were all the same. Taking the absorption figure of the product of the reaction with 0.9 *M* benzoate as the final limit, a nomogram was obtained (Fig. 3) for the calculation of percentage formation of hemichrome. The percentages of hemichrome formation calculated from each of the nomograms at any wave lengths were all in good agreement. Thus the percentages of hemichrome formation at every benzoate concentration were all calculated from the nomogram of the wavelength 535mμ. These values were plotted against log concentrations of benzoate as shown in Fig. 4. A sigmoid curve of a high order reaction was thus recorded. The percentages of hemichrome formation in each benzoate concentration remained unaffected by temperature changes between 18° and 22°. They are also independent of the concentrations of methemoglobin (0.05 and 0.1 per cent). The reaction rate was, however, dependent on the benzoate concentration; the rates were higher at higher benzoate concentrations (Fig. 5). The reaction came to interruption after certain levels of hemichrome formation

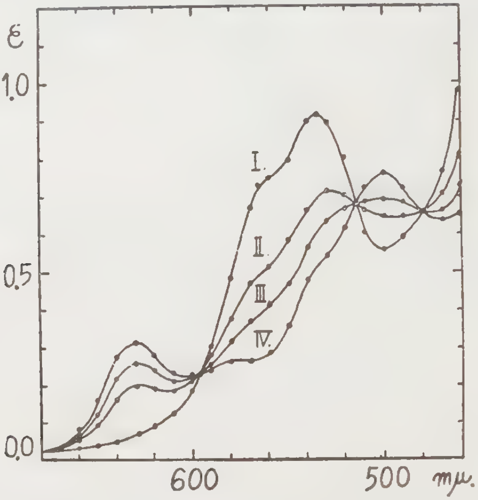
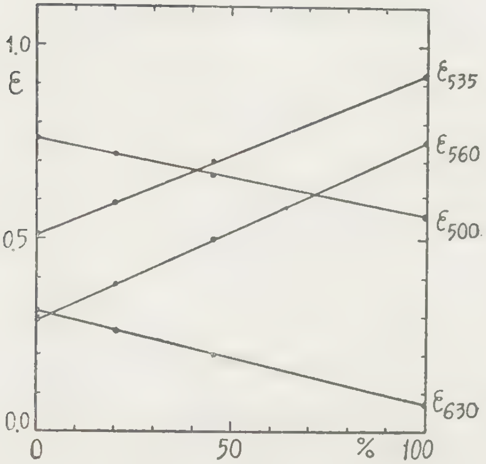
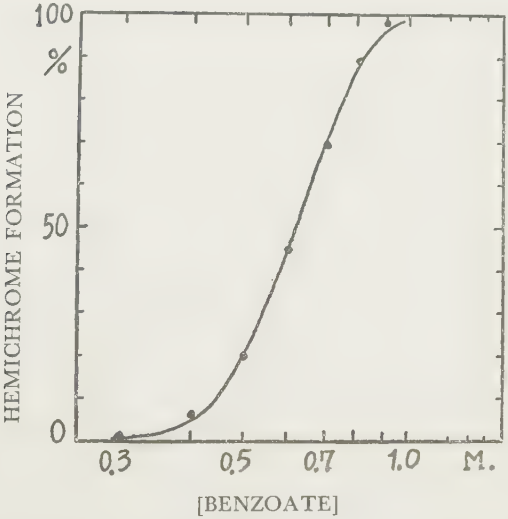


FIG. 2. The absorption figures obtained by addition of varying benzoate concentrations.
pH: 7.2
Concentration of methemoglobin: 0.1%
Concentrations of benzoate: I, 0.9 *M.*, 1.0 *M.* and 1.2 *M.*; II, 0.6 *M.*; III, 0.5 *M.*; IV, 0.2 *M.* and 0.0 *M.*



HEMICHROME FORMATION

Fig. 3. Nomogram for the calculation of percentage of hemichrome formed (obtained from Fig. 2.)



[BENZOATE]

Fig. 4. Plotts of the percentage of hemichrome formation vs log concentrations of benzoate.

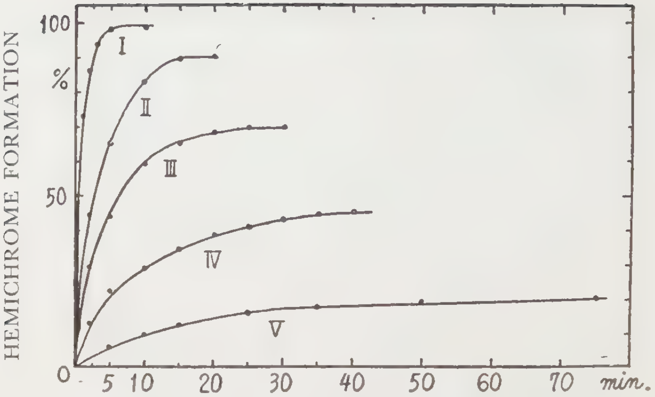


Fig. 5. Curves representing the reaction rate of hemichrome formation by benzoate of varied concentrations.
pH: 7.2. Temperature: 18°
Concentration of methemoglobin: 0.1%.
Concentrations of benzoate: I, 0.9 *M.*; II, 0.8 *M.*; III, 0.7 *M.*; IV, 0.6 *M.*; V, 0.5 *M.*

was attained in correspondence to the concentrations of benzoate applied. When each of these reaction solutions at their final stage was diluted with distilled water until the benzoate concentration in each solution became 0.1 *M* the pigment in each of them could be completely restored to methemoglobin.

The experimental observations so far described indicate that the reaction of methemoglobin with benzoate in the experimented concentration range is a completely reversible one, and that the reaction system consists simply of two components: methemoglobin and hemichrome. The relation between log concentration of benzoate and percentages of hemichrome formed could be expressed by a sigmoid curve of high order reaction. In view of this fact, the absorption change induced by benzoate seems not to be due to a simple coordinative linking of benzoate to heme-Fe. More probably, an equilibrium is established between two components: one of which is the native methemoglobin and the other is the structurally modified methemoglobin, *i.e.* a sort of hemichrome. The latter has been produced by the action of benzoate upon methemoglobin through a slight and reversible modification of globin molecule (unfolding or dislocation of some Fe-linking groups from peptide chain of globin) so that a globin-N has become freely reactive and capable of coordination to heme-Fe from its both sides.

Now, when the pigment solution produced by the reaction with benzoate was reduced with $Na_2S_2O_4$, an absorption change was observed as shown in Fig. 6, III; the resulted figure was identical with that of reduced hemoglobin. At the instant of the addition of $Na_2S_2O_4$, however, an absorption figure similar to that of hemochrome with its maxima at 562 and 530 mμ could be observed, which were transient but

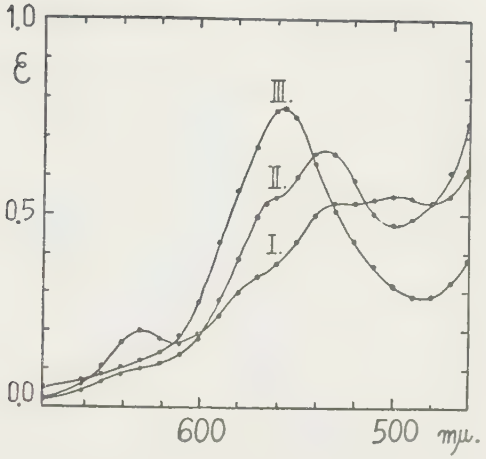


Fig. 6. The absorption change of hemichrome formed by benzoate on reduction with $Na_2S_2O_4$.

I. Methemoglobin + benzoate (0.6 *M*).

II. Methemoglobin + benzoate (0.8 *M*).

III. On reduction of I and II, with $Na_2S_2O_4$.

clearly distinct. The figure was extremely labile, changing in a short time to that of reduced hemoglobin, so that an exact determination of this transient hemochrome figure could not be undertaken. This fact is of extreme interest, because it indicates that the effectiveness of benzoate is controlled by the electric charge of heme-Fe. The structural modification of globin part induced by benzoate in Fe^{+++} state can easily be reconverted into the native structure when the pigment is reduced to its Fe^{++} state. Globin structure seems to be more resistent against benzoate in the Fe^{++} state of heme part than in its Fe^{+++} state. The fact was further studied in the next section.

II. Reaction between Benzoate and Reduced Hemoglobin

Oxyhemoglobin was reduced to hemoglobin by addition of $Na_2S_2O_4$ in the smallest effective amount. To this hemoglobin solution, benzoate was added in its final concentration above 1.0 *M*. The absorption figure of the solution cnanged gradually with increasing benzoate concentration (Fig. 7). As shown in Fig. 7, isosbestic points were clearly demonstrated in the change of absorption, indicating the existence of two component systems of the absorption. Further it was proved that the absorption change in this system in the experimented concentration range of benzoate is completely reversible. The system established was the equilibrium between reduced hemoglobin and hemochrome. As shown in Fig. 8, a sigmoid curve of high order reaction was obtained on plotting the log concentrations of benzoate against percentages of hemochrome formed, the latter being calculated by the same principle as described in *Section I.*

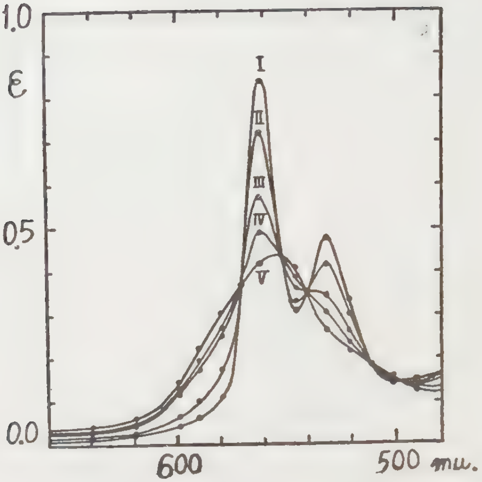


Fig. 7. Changes of the absorption figures of reduced hemoglobin on varying benzoate concentrations.

Concentration of reduced hemoglobin: 0.06%.

Concentrations of benzoate: I, 1.8–2.0 *M*; II, 1.5 *M*; III, 1.35 *M*; IV, 1.3 *M*; V, 1.0 *M*, 0.9 *M* and 0.0 *M*.

When hemichrome formed by treatment with benzoate above 1.0 *M* was reduced by $Na_2S_2O_4$, an absorption figure of hemochrome was obtained. This was the same figure of the pigment as was formed from reduced hemoglobin by treatment with benzoate. With benzoate in its final concentration above 2.0 *M*, the hemochrome figure obtained reached its maximum limit. So it is most probable that the transient hemochrome figure observed at the instant of $Na_2S_2O_4$ reduction of the reaction product of methemoglobin formed by 0.6–0.8 *M* benzoate, is identical with that of hemochrome produced directly from hemoglobin by benzoate. The absorption figure of the pigment formed from reduced hemoglobin by benzoate is, as understandable from the figure, markedly similar to those of pyridine hemochrome or of alkali denatured globin hemochrome. Thus methemoglobin and reduced hemoglobin seemed to be effected by benzoate in a similar manner at their globin part both giving a similar change in absorptions. The resulted absorptions were qualitatively identical with those of the compounds in which their heme-Fe (Fe^{++} or Fe^{+++}) are coordinatively combined with pyridine from both sides of Fe. Further support was thus given for the assumption that the globin part of these heme-proteins was structurally modified by benzoate to make their globin-N freely reactive (unfolded or dislocated from their peptide chains) to coordinate to the heme-Fe from its both sides.

III. Effect of Glycerol upon the Reaction of the Hemichrome Formation

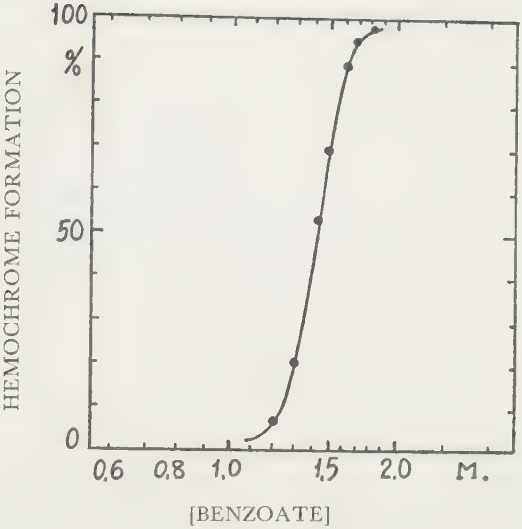


Fig. 8. Plotts of the percentage of hemochrome formation vs log concentrations of benzoate.

It was generally assumed that glycerol combines with protein on its surface, so that it protects protein from denaturation caused by various denaturants. Therefore, the possible effect of glycerol upon the reaction system of hemoglobin and benzoate was further studied. With the addition of glycerol (40 per cent) to methemoglobin solution, no absorption change was observed (Fig. 9, I). When benzoate was further added to the solution to its final concentration of 0.9 *M*, an absorption change occurred as shown in Fig. 9, III. With this benzoate concentration without the addition of glycerol, the solution gave the absorption figure shown in Fig. 9, II. In these figures, too, isosbestic points were clearly demonstrated. The absorption change of hemichrome solution through benzoate in the presence of glycerol seems, thus, to be a result of an equilibrium shift of the system. With the addition of 40 per cent glycerol, the percentage of hemichrome formation decreased to about 1 per cent. When glycerol was added to the solution of Fig. 9, II or when methemoglobin solution was mixed with glycerol and benzoate was then added to it in its final concentration of 0.9 *M*, both gave an absorption figure identical with that given in Fig. 9, III. It seems to be probable that glycerol, by combination with protein, protects globin from the affection by benzoate. The nature of the combination remains, however, as yet unclarified.

IV. Effect of Heat upon the Absorption Figure of the Hemichrome

Methemoglobin was completely converted to hemichrome by the addition of benzoate to 0.9 *M* (Fig. 10, I). By heating at 90°, the absorption figure I changed to that of II. By cooling, the figure II was reversed promptly to figure I. It is inter-

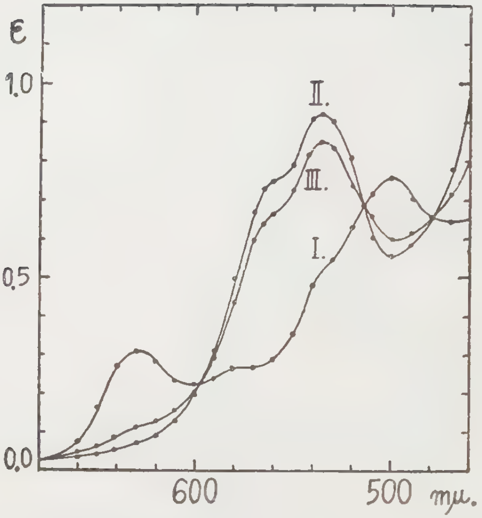


Fig. 9. Absorption changes of hemichrome by benzoate in the presence of glycerol.

I. Methemoglobin, methemoglobin+glycerol (40%).

II. Hemichrome. (0.9 *M* benzoate).

III. II+glycerol (40%)

esting to note that there occurred no precipitation during this conversion. Generally, heat coagulation of hemoglobin occurs most easily at about pH 7.0. From the fact that no heat coagulation of hemoglobin occurred when benzoate was added, it may be assumed that benzoate combines with globin on its surface preventing its intermolecular aggregation.

From the experimental results so far obtained it may be inferred conclusively that benzoate combines multimolecularly and reversibly with globin molecule causing a configurational modification of the latter in such a way as to enable its globin-N existing near the heme-Fe to coordinate with the latter.

SUMMARY

1. Methemoglobin can be converted to a hemichrome like compound through the reaction with benzoate. Absorption figure of this hemichrome derivative is similar to that of the related compound obtained by the reaction with salicylate. Essentially they are similar to that of pyridine hemichrome.

2. Reduced hemoglobin was converted into a hemochrome like

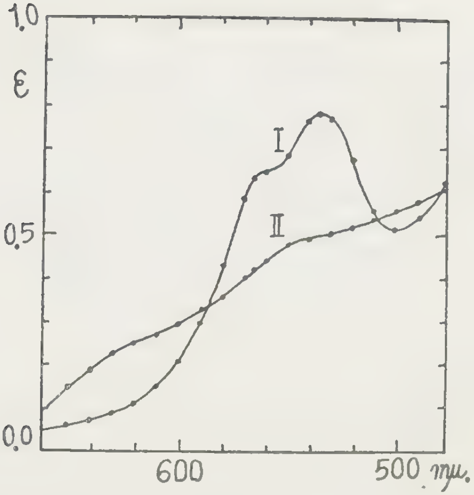


Fig. 10. The absorption change of hemichrome on heating.
I. Methemoglobin + benzoate (0.9 *M*).
II. On heating at 90°, this is reversible on cooling.

compound by benzoate. The latter compound gave absorption spectra similar to those of alkali denatured globin hemochrome and of pyridine hemochrome.

3. The relation between the percentage of hemichrome resp. hemochrome formation and the benzoate concentration could be expressed by a sigmoid curve of high order reaction.

4. Much higher concentration of bezoate was needed for the conversion of reduced hemoglobin than for that of methemoglobin. In the concentration of benzoate below 1.0 *M*, the hemichrome formed could be quantitatively converted into reduced hemoglobin through reduction by $Na_2S_2O_4$. The conversion proceeded through a hemochrome like substance as an intermediary compound which could be observed only transitorily.

5. Glycerol exerted inhibitory effect upon the reaction of hemichrome formation by benzoate.

6. The hemichrome like substance formed by benzoate could not be precipitated on heating. This hemichrome like compound showed a reversible spectral displacement on heating.

I should like to express my thanks to Prof. K. Kaziro and Dr. G. Kikuchi for their interest and helpful discussion during the course of this work.

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ON THE REACTION PROCESS OF PSEUDOHEMOGLOBIN FORMATION

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(Received for publication, October 23, 1953)

Choleglobin is formed in the reaction system of hemoglobin, ascorbic acid and oxygen through the possible direct participation of hydrogen peroxide generated in the given system (*1–2*). Its determinant role was experimentally evidenced throughout the studies so far reported from our laboratory (*2*). As the matter of fact, choleglobin can be directly formed from hemoglobin by the addition of H_2O_2 to the latter without the presence of ascorbic acid.

On the other hand, as reported by Barkan (*3*), when H_2O_2 was added to hemoglobin in the presence of KCN, pseudohemoglobin was formed which has been assumed by him to be structurally intimate to choleglobin. Barkan obtained further a substance showing the absorption spectrum very similar to pseudohemoglobin by the action of hydrazine and oxygen on hemoglobin in the presence of KCN. From these, it may be pointed out that KCN seems to be indispensable in the pseudohemoglobin forming reaction system. As to the nature of the participation of KCN in the mentioned reaction, however, almost nothing is known. Also the chemical relation between choleglobin and pseudohemoglobin is not yet clear.

Our present communication deals with the quantitative observations made on the reaction of pseudohemoglobin formation under the various conditions intending to study the role of KCN in the system.

EXPERIMENTAL

For the present experiment, the hemoglobin solution deficient in catalase was mainly used which was obtained from the erythrocytes of a patient "*Anenzymia catalasea*"(*4*). In some cases in which sufficiently higher amounts of KCN were used, however, hemolyzate of rabbit erythrocytes was used. The maximum concentration of KCN used was 1 *M*. For the dilution of KCN, 1 *N* solution of K_2CO_3 was used instead of water in order to avoid the change of pH of the reaction mixture.

Ascorbic acid was dissolved in redistilled water every time at use and was neutralized with equivalent NaOH. "Spekker" spectrophotometer was used for the determination of the optical densities. All the experiments were undertaken at room temperature.

RESULTS

I. Reaction of the HbO_2—KCN—H_2O_2 system

Changes of the Absorption Figure during the Reaction Process—Reactions and the optical measurements were carried out directly in the absorption cell. By the addition of H_2O_2 to HbO_2 solution in the presence of KCN, the absorption spectrum of HbO_2 diminished rapidly. The conversion of the absorption figure completed after 5–7 minutes: the final figure is shown in Fig. 1, I. Absorption maximum in the green was at 540 mμ in agreement with that of CN-metHb. In the red region, however,

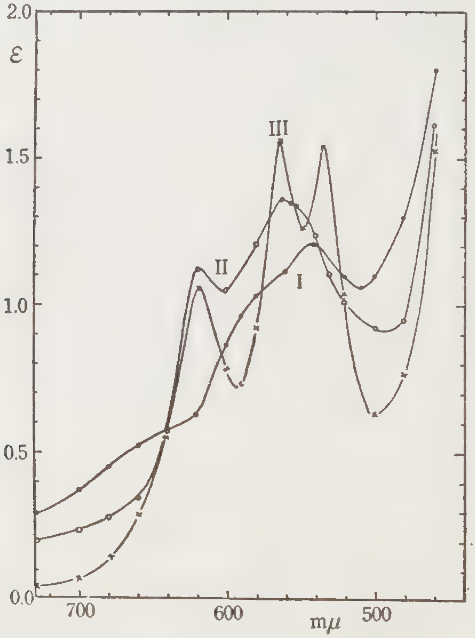

Fig. 1. Changes of the absorption figure in the reaction of HbO_2—KCN—H_2O_2 system.

4.0 ml. of 0.25% HbO_2(pH 7.38), 0.5 ml. of 1 *M* KCN and 0.5 ml. of 0.01 *M* H_2O_2. I, at 7 minutes after the reaction started; II, I+$Na_2S_2O_4$; III, II+0.5 ml. of 0.6% NaOH (absorption figure was corrected according to the dilution) the figure differed from that of CN-metHb and showed a diffuse absorption without any significant maximum. When the reaction solution represented by Fig. 1, I was reduced by the addition of a small amount of powdered $Na_2S_2O_4$, its absorption curve changed to Fig. 1, II. The absorption in the green was that of reduced Hb, but in the red region there appeared distinctly a new absorption maximum at 618 mμ. Now, NaOH was added finally to the above solution, so that the globin part of the pigment was brought to complete denaturation. An absorption figure was then obtained as shown in Fig. 1, III. As seen from Fig 1, III, the absorption maximum in the red region showed no desplacement from 618 mμ even the globin part of the pigment has been completely denatured.

Though the reaction condition described in Fig. 1 was found to be most suitable for the pseudohemoglobin formation as described in the following Experiments 2) and 3), a considerable amount of the protoheme remained as yet unaffected in the solution. Namely the spectrum in the green region in Fig. 1, III, can be rendered mainly to that of alkali denatured globin hemochrome, some of them being substituted by CN^-. On this point, some discussion will be made later.

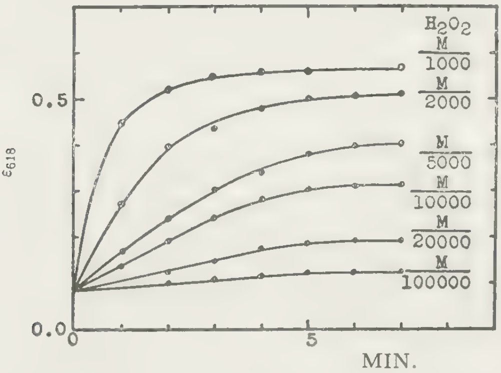

FIG. 2. Effect of the amounts of H_2O_2.

4.0 ml. of 0.25% HbO_2 (pH 7.38), 0.5 ml. of 1 *M* KCN and 0.5 ml. of H_2O_2 of varied concentrations. The concentrations designated in the figure represent the final concentrations of H_2O_2 in the reaction mixtures.

2) *Influence of the Amounts of Hydrogen Peroxide Added*—A series of experiments with varied amount of H_2O_2 were carried out under the conditions described in Fig. 2. The changes of ε_{630} were followed during 7 minutes and used as the measure of reaction velocity of pseudohemoglobin formation. The results were recorded by the curves in Fig. 2. Both the reaction rate and the final amounts of pseudohemoglobin formed increased with the increasing amounts of H_2O_2 added.

3) *Influence of the Amounts of KCN Added*—A definite amount of H_2O_2 was added

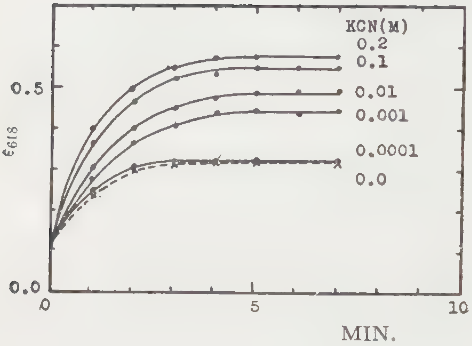

Fig. 3. Effect of KCN.

4.0 ml. of 0.25% HbO_2 (pH 7.38), 0.5 ml. of KCN of varied concentrations and 0.5 ml. of 0.005 M H_2O_2. The numbers in the figure represent the final concentrations of KCN.

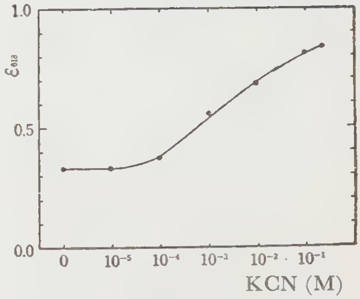

Fig. 4. Relationship between the KCN concentration and the amount of pseudohemoglobin formed.

The same reaction solutions described in Fig. 3 were treated NaOH and then with $Na_2S_2O_4$ and ε_{618} of them were measured.

to each reaction mixture containing KCN in varied concentrations and the increases of ε_{630} were followed up with the time. The results are shown in Fig. 3. Both the reaction rate and the final amounts of pseudohemoglobin formed increased with increasing amount of KCN added. The relationship between the concentration of KCN and the optical density at 618 mμ of the reaction mixture after the treatment with NaOH and $Na_2S_2O_4$ is shown in Fig. 4. It is quite obvious that KCN accelerates the formation of pseudohemoglobin.

II. Reaction in the System of Hb, KCN, Ascorbic Acid and Oxygen

1) *Changes in the Absorption Figure*—The reaction mixture consisted in 4.0 ml. of 0.25 per cent HbO_2, 0.5 ml. of 1 M KCN and 0.5 ml. of 0.05 M ascorbic acid. Each of the above solutions was individually pipetted into the absorption cell following described order, mixed immediately and was kept on standing as to be reacted with atmospheric oxygen. The absorption spectrum of HbO_2 of the reaction mixture diminished gradually and instead, there arose merely a single absorption maximum in the green (Fig. 5, IV). The conversion completed after 15 to 20 minutes. The absorption curve of Fig. 5, IV is qualitatively similar with that of Fig. 1, I. Fig. 6 is the absorption figures of the reaction solutions observed after the treatment with NaOH and $Na_2S_2O_4$ at certain intervals of reaction time. New absorption arose sharply in

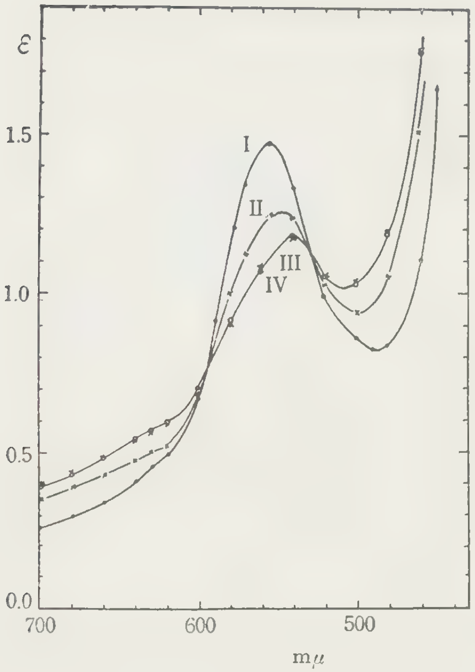

Fig. 5. Absorption figures at the final stages of the reaction of Hb—KCN—ascorbic acid—Oxygen system with varied concentrations of KCN.

4.0 ml. of 0.25% HbO_2 (pH 7.38), 0.5 ml. of KCN and 0.05 *M* ascorbic acid. The concentrations of KCN in *M* (mole per liter) : I, O ; II, 10^{-3} ; III, 10^{-2} ; IV, 10^{-1}. T=22°.

the red at 618 mμ, while the absorptions in the green decreased, isosbestic points being clearly demonstrated. After 20 minutes, no further absorption change were observable.

2) *Influence of the Amounts of Ascorbic Acid*—A series of experiments with varied concentrations of ascorbic acid was carried out. Each reaction mixture was treated (with NaOH and $Na_2S_2O_4$ at certain intervals of reaction time and the increase of ε_{618} was measured. The results are shown in Fig. 7. As seen from Fig. 7, the reaction rate increased with the increase of ascorbic acid concentration. In the reactions with relatively lower concentrations of ascorbic acid, the reaction curves were S-shaped. The maximum levels of ε_{618} obtained in each experiment with varying ascorbic acid concentrations, however, remained almost constant as 0.76–0.78.

3) *Influence of the Concentrations of KCN*—All the HbO_2 solutions used in the experiments in this section were free from catalase.

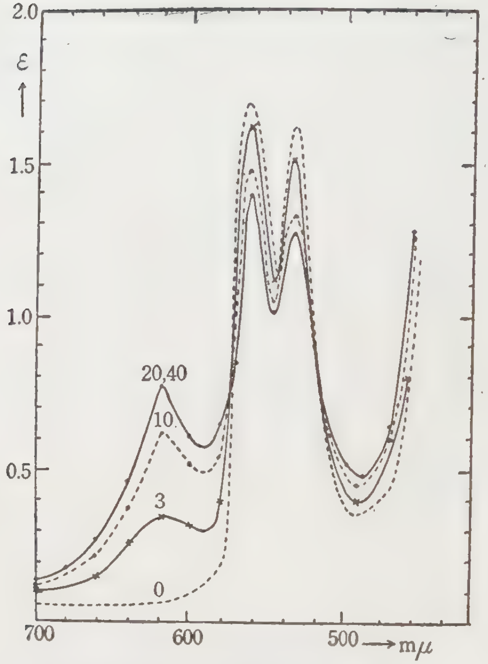

FIG. 6. Formation of pseudohemoglobin after each intervals of time.

16 ml. of 0.25% HbO_2 (pH 7.38), 2 ml. of 1 *M* KCN and 2 ml. of 0.1 *M* ascorbic acid; T=13°.

2.5 ml. of the reaction mixture was taken out after each intervals of time as designated in the figure and 0.5 ml. of 6% NaOH and a small amount of powdered $Na_2S_2O_4$ were added to it and then the absorption curve was measured.

i) Changes of the absorption figures: In each of the experiment solutions with the addition of different amounts of KCN, the spectrum of HbO_2 diminished as the reaction proceeded. The final spectra are shown in Fig. 5. Diffuse absorption arose in the red region and decrease and displacement of absorption maximum was observed in the green region corresponding to the increase of KCN added. Fig. 5, I was obtained from the experiment in which K_2CO_3 was used in place of KCN; the absorption maximum was at 555 mμ and the general feature of the absorption curve in the green region is similar to that reduced Hb. As the concentration of KCN in the reaction mixture was increased, the position of the absorption maximum shifted toward the short wave length side until it was finally fixed at 540 mμ, the absorption maximum

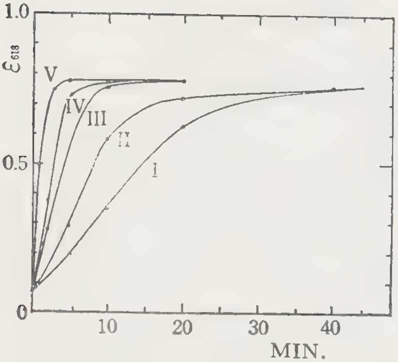

FIG. 7. Influence of the amounts of ascorbic acid.

Reaction conditions and the procedures of measurement were the same as those of Fig. 5. Final concentrations of ascorbic acid in $10^{-2}M$: I, 0.25; II, 0.5; III, 1.0; IV, 2.0; and V, 4.0.

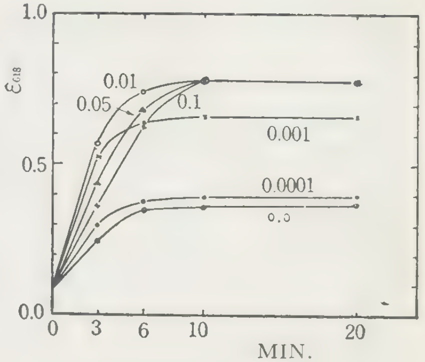

FIG. 8. Effect of KCN.
2.0 ml. of 0.25% HbO_2(pH 7.38), 0.25 ml. of KCN and 0.25 ml. of 0.1 *M* ascorbic acid. T=22°. The numbers in the figure represent the final concentrations of KCN in *M* (mole per liter).

corresponding to that of CN-metHb as described already.

ii) Follow up of the reaction process: As shown in Fig. 8, with the increase of KCN added, both the reaction rate and the final value of ε_{618} increased in each experiment. When the concentration of KCN in the reaction mixture exceeded over a ~~certain~~ limit, however, the reaction rate, especially of the initial stage, decreased, so that a S-shaped reaction curve was formed. When the increase of ε_{630} of the reaction mixture was directly measured instead of ε_{618} without the treatment of the solution by NaOH and $Na_2S_2O_2$, the sigmoid feature of the reaction curve was more distinguished and was recognizable even when the reaction was carried out under the addition of K_2CO_3 in place of KCN.

As shown in Fig. 8, the maximum value of ε_{618} did not exceed 0.78 even when the concentration of KCN was sufficiently high. The fact would be greatly related to that observed in the experiment (Fig. 7) in which the concentration of ascorbic acid was varied.

When KCN was further added to the solution added with K_2CO_3 instead of KCN, 10 minutes after the reaction started (at this time the reaction has been reached to its final stage in this reaction condition), the green pigment formation immediately restored. The reaction rate and the final amount of green pigment formed increased in parallel with the amount of KCN further added. In this case, however, the maximum value of ε_{618} could not be reached to 0.78.

iii) Oxygen consumption in the course of the reaction process: Oxygen consumption was measured by Warburg's manometer along with the reaction process.

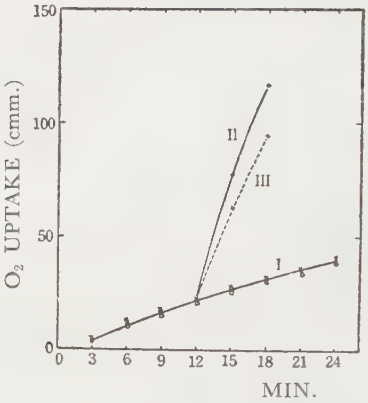

FIG. 9. Oxygen consumption during the reaction process. 2.0 ml. of 0.25% HbO_2 (pH 7.38), 0.25 ml. of KCN and 0.25 ml. of 0.05 M ascorbic acid. T=25°. Final concentrations of KCN in M: ○—○ 10^{-3}, ×—× 10^{-2}, ●—● 10^{-1}.

I, measured without shaking of manometer; II, manometer was shaken at the intermediate of the reaction; III, control experiment to II with phosphate buffer in place of HbO_2 solution.

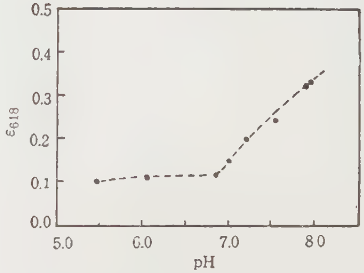

Fig. 10. Effect of pH.

0.6 ml. of 1% HbO_2, 1.8 ml. of M/15 phosphate buffer in varied pH, 0.3 ml. of 0.2 M KCN and 0.3 ml. of 0.2 M ascorbic acid. In this experiment, the optical densities of the reaction solutions were measured directly without treatment by NaOH and $Na_2S_2O_4$ at 618 mμ. All the determinations were undertaken after 5 minutes of the reaction.

As shown in Fig. 9, the rate of the oxygen consumption was extremely small when the manometer vessel stood without shaking and the rate was scarsely influenced by the concentration of KCN. In some of the experiments the manometers were shaked in the middle of the reaction, then the consumption of oxygen took place vigorously by shaking. Even in these cases, however, the rate of oxygen consumption was almost independent from the concentration of KCN in the reaction mixture.

Thus the effect of KCN in favour of the pseudohemoglobin formation seems not to be based on its effect upon the reaction rate of the ascorbic acid oxidation, but probably on some other reasons.

4) *Effect of pH*—As shown in Fig. 10, the reaction rate of pseudohemoglobin formation increased as the rise of pH of the reaction solution especially in the alkaline side.

DISCUSSION

Our above findings may serve some explanation on the mechanism of the pseudohemoglobin formation from oxyhemoglobin under the effects of KCN and H_2O_2. It is remarkable to have found that the maximum amount of pseudohemoglobin finally formed was not influenced by the amount of ascorbic acid. The reaction came to end at much lower level of pseudohemoglobin than that theoretically expected. Similar relations can be pointed out in the reactions in *Experiment II*, 3), ii) (Fig. 8), also even in *Experiment I*, 2) and *I*, 3). The lower yield of pseudohemoglobin can possibly be explained as follows: methemoglobin which is formed in this reaction system combines with CN^- to form CN-methemoglobin and since the latter compound can hardly be reduced by ascorbic acid, that portion of hemoglobin which has been converted to CN-metHb may be excluded from the reaction system of pseudohemoglobin formation. The maximum level of pseudohemoglobin formation has been proved to be almost constant independently of the ascorbic concentrations. Thus a definite ratio has been established between the formation of pseudohemoglobin and metHb in the reaction process, both substances being formed through a common step reaction. This is a remarkable feature of the present reaction for which our explanation will be reserved for future.

The effect of pH on the pseudohemoglobin formation was great. The formation of pseudohemoglobin remained fairly small unless the pH of the reaction solution became relatively high. This suggests the necessity of ionic CN^- rather than HCN for the pseudohemoglobin formation.

It is noteworthy too that when K_2CO_3 was used in place of KCN, the green pigment formation was interrupted at its lower level, and the absorption spectrum in the green region then agreed with that of reduced hemoglobin. This suggests that the affinity of Hb to molecular oxygen should have been fairly decreased in this system. By the addition of KCN or K_2CO_3 to HbO_2 without ascorbic acid, however, HbO_2 would not easily be converted to reduced Hb. It should not be overlooked that the reaction system of Hb—K_2CO_3—ascorbic acid—oxygen is the same to that of choleglobin formation except the higher pH in the former system caused by K_2CO_3. The nature of the reaction in the presence of K_2CO_3 seems thus to be different from that of the ordinary choleglobin formation in some points. For instance, the rate of the green pigment formation was extremely high only within the earlier stage of the present reaction. And after that the amount of green pigment reached to a definite value, the reaction was interrupted though a sufficient amount of ascorbic acid was still remaining in the reaction mixture as to be able to reduce the metHb formed during the process of the reaction. (The reaction however, revived later again and the rate curve arose again after longer intervals of time).

The reaction product of the earlier stage of this reaction seemed to differ from choleglobin. The heme-iron of the former was not easily splittable by the treatment with hydrochloric acid (detailed experiments will appear in the next report).

The true mechanism of the participation of KCN in the reaction of pseudohemoglobin formation remains as yet unclarified in our present experiments. It may be probable at present to assume that KCN affects globin molecule in hemoglobin into some sort of structural modifications.

SUMMARY

The reaction process of pseudohemoglobin formation was studied quantitatively by means of spectrometrical observations.

1. The yields of pseudohemoglobin in the system of HbO_2—KCN—H_2O_2 increased with increase of H_2O_2 or of KCN. Its maximum yield, however, never exceeded over a determinated limit.

2. Pseudohemoglobin was proved to be formed also in the reaction system of Hb—KCN—ascorbic acid—O_2. Also in this system, the production of pseudohemoglobin reached to its maximum level relatively in short time and the reaction was then interrupted remaining large quantity of unaffected protoheme in the form of CN-metHb. In the presence of sufficient amount of KCN, the rate of pseudohemoglobin formation increased with increase of ascorbic acid added. But, the final yield of the pigment never exceeded over a certain limit. It was suggested thus, hemoglobin was converted in this reaction system into pseudohemoglobin and methemoglobin and in constant proportion of these two products.

3. With KCN of extremely lower concentrations, the final yield of pseudohemoglobin also decreased.

4. It seemed within the reaction process of pseudohemoglobin formation a slight denaturation has been occurred in the molecule of globin and the O_2-binding capacity of hemoglobin was proved to be lowered within this reaction process.

5. For the reaction of pseudohemoglobin formation, pH values above 7.0 were afforded as favorable. It was suggested thus that CN^- is effective in the present reaction, but not HCN.

The present investigation was supported by Prof. K. Kaziro, for which the authors wish to thank. The technical assistance of Dr. T. Tomimura in manometrical observations is also acknowledged with pleasure.

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ON THE DETACHMENT OF IRON FROM PSEUDOHEMOGLOBIN

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Pseudohemoglobin was obtained firstly by Barkan (*1*) from HbO_2 through the action of H_2O_2 in the presence of KCN. Barkan reported further that the iron in pseudohemoglobin can easily be split out from its molecule by treatment with 0.4 per cent hydrochloric acid and designated this sort of iron as "easily splittable iron". On the other hand, the iron in verdohemochrome and choleglobin can also be easily splitted out from their molecule by the same procedure (*2*). The relation of the chemical structure between pseudoheme, verdoheme and choleheme remains as yet unclarified. Only a suggestive interpretation on this problem has been presented by Lemberg (*3*) recently. In one of our papers (*4*), some experimental results and discussions on the splitting of iron from choleglobin were presented previously. Also in our another paper (*5*), the results of quantitative follow up of the reaction process of pseudohemoglobin formation under the various conditions were reported. The present work deals with more intimate study on the detachment of iron from pseudohemoglobin and related compounds to make more extensive analyses of the chemical relation between these green pigments mentioned above.

EXPERIMENTAL METHODS

Material—The hemoglobin solution used in our present experiment was without catalase. The blood sample was obtained from a patient known as "*Anenzymia catalasea*" (*6*). Solutions of ascorbic acid and KCN were prepared as described in the previous paper.

Reaction Series—All of the experiments were carried out in small beakers and in parallel series for the spectral observations of green pigment formation at each time intervals and the determinations of detachable iron in the green pigment formed.

Procedure for the Measurement—"Spekker" spectrophotometer was used for the measurement of the green pigment formation. For the spectrophotometrical measurement, the reaction mixture was treated with NaOH and $Na_2S_2O_4$ at each intervals of time and the optical density of the solution at 618 mμ was measured which corresponds to the absorption maximum of the green pigment thus treated. Detailed procedure has been presented in our previous paper. For the measurement of the detached iron, *o*-phenanthroline method of Barkan was used after some modifications on account of the presence of KCN or K_2CO_3 in the reaction mixture and of higher pH of the reaction mixture in some of the experiments. Moreover the complex formation of iron with phenanthroline can be hindered to certain extent by KCN. Accordingly the following procedure was used: 2.5 ml. of 0.618 *M* HCl was added to the reaction mixture, mixed well, and 1.5 ml. of the content was then transferred into a centrifuging tube. The tube was plugged closely with a rubber stopper and was kept on standing at 37° for one hour and then at the room temperature for about 24 hours. The reaction mixture was then deproteinized by the addition of 1 ml. of 20 per cent trichloracetic acid, centrifuged, and 1 ml. of the supernatant was transfered into a small test-tube with a mark line of exactly 2 ml. After the addition of 2 drops of concentrated pure H_2SO_4 and 4 drops of perchloric acid, the test-tube was heated over a little flame until the oxidation was completed. The content was neutralized with saturated NaOH and diluted with redistilled water until to the mark line of the test-tube. One ml. of the content thus obtained was used for the determination of iron. Detailed procedure for the iron determination by *o*-phenanthroline method was described in our previous paper.

Calculation of the Detachable Iron Content—The final HbO_2 concentration in every

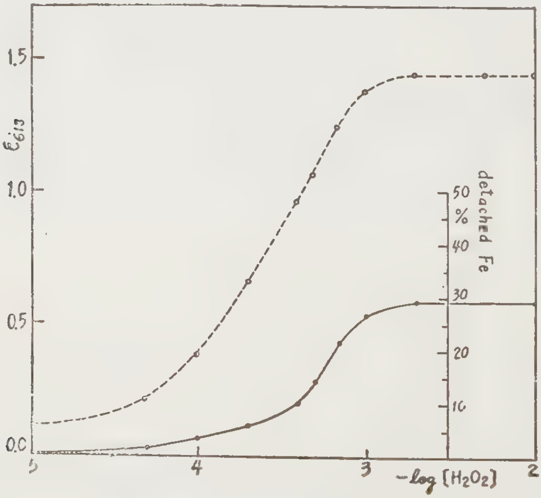

FIG. 1. Formation of pseudohemoglobin and increase of the detachable iron content in the reaction of HbO_2–KCN–H_2O_2 system. Reaction mixtures: 4 ml. of 0.5% HbO_2 (pH 7.38), 0.5 ml. of KCN and 0.5 ml. of H_2O_2 in varied concentrations. Full line: detachable iron; broken line: pseudohemoglobin.

reaction mixtures was 0.4%. Providing the molecular weight of hemoglobin as 67,000, the heme-iron in 9.4 per cent HbO_2 solution corresponds to 1313 γ per cent. The detachable iron content was expressed in the figures by the percentage of detached iron experimentally found to the total heme-iron calculated.

RESULTS

I. Reaction of the HbO_2—KCN—H_2O_2 System

The formation of pseudohemoglobin was studied in the system under the condition described in Fig. 1 and with varying concentrations of H_2O_2. The results are given in Fig. 1, continuous curve indicating the formation of pseudohemoglobin and broken curve indicating the detachable iron content increased with increase of the concentration of H_2O_2 added.

II. Reaction of the Hb—KCN—Ascorbic Acid—O_2 System

Experiments at Room Temperature—2.0 ml. of 0.5 per cent HbO_2, 0.25 ml. of KCN

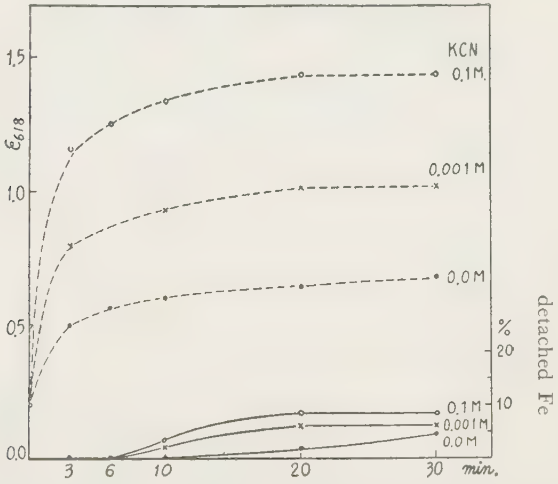

FIG. 2. Formation of pseudohemoglobin and increase of the detachable iron content in the reaction of Hb—KCN—ascorbic acid—O_2 system at room temperature.
Reaction mixtures: 2.0 ml. of 0.5% HbO_2, 0.25 ml. of KCN in varied concentrations and 0.25 ml. of 0.1 *M* ascorbic acid. Full line: detachable iron; broken line: pseudohemoglobin. Designated numbers in the figure represent the final concentrations of KCN in *M* (*mole per liter*).

in varied concentrations and 0.25 ml. of 0.1 *M* ascorbic acid neutralized with equivalent NaOH were pipetted into each of the several beakers. The mixtures were kept on standing to be reacted by atmospheric oxygen. The formation of green pigment and the content of detachable iron were determined. The results are given in Fig. 2. As seen from Fig. 2, the formation of green pigment increased obviously with the increase of the concentration of KCN, while the content of detachable iron remained extremely in lower level. The liberation of iron was not in accord with the green pigment formation, its increase began much later than the latter. From the content of detachable iron, moreover, the effect of KCN could hardly be recognized. In Fig. 3, the results of the prolonged experiments are given. As shown in the figure, both the green pigment formation and the increase of the content of detachable iron reached to their maximum values after about 30 minutes and they showed no increase later.

Comparing with the fact that the maximum values of the detachable iron content in the experiment series shown in Figs. 2 and 3 were found as low as about 10% of the total heme-iron, while the maximum value of that was proved to reach as high as about 30 per cent in the reaction of the HbO_2—KCN—H_2O_2 system. In cases with larger amounts of H_2O_2, it would not be overlooked the possible contamination of a few amounts of iron derived from a sort of by-reaction.

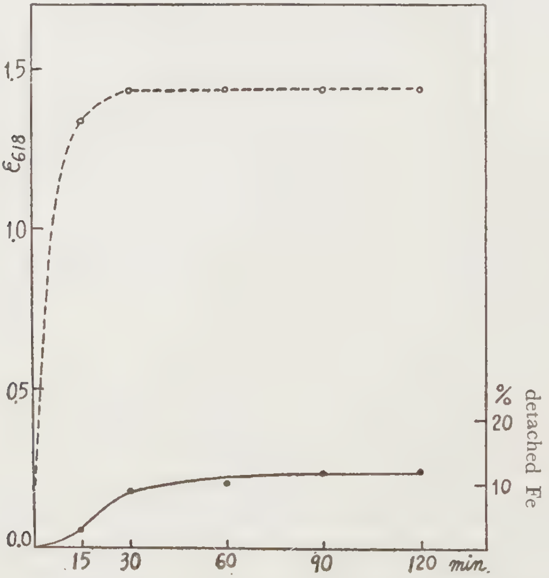

Fig. 3. Reaction process of Hb—KCN—ascorbic acid—O_2 system observed in prolonged time scale.
Final concentrations of KCN: 0.1 *M*. Other reaction conditions and the designations in the figure are the same as in Fig. 2.

Experiments at 38°—The results of the experiments carried out at 38° are given in Fig. 4. In these experiments, also, the amounts of liberated iron was found very low. It is to be pointed out in Fig. 4 that while the liberation of iron was almost negligible in the experiments at room temperature when K_2CO_3 was used in place of KCN, the yields of liberated iron was found to be significantly high in the reaction at 38°. It was proved to increase steadily along with the reaction proceeded.

Effect of KCN Added Intermediately during the Reaction of Choleglobin Formation—When KCN was added into the reaction solution of choleglobin formation intermediately during the reaction in progress, the value of ε_{618} increased promptly and the yield of liberated iron accordingly increased to certain extent, but only during a short time. The reaction reached soon to its maximum value.

III. *Reaction of the Hb—K_2CO_3—Ascorbic Acid—O_2 system*

It was pointed out in the previous paper that the green reaction products in the Hb—K_2CO_3—H_2O_2 system as well in the Hb—K_2CO_3—ascorbic acid—O_2 system might be in close relation to pseudohemoglobin produced in the presence of KCN.

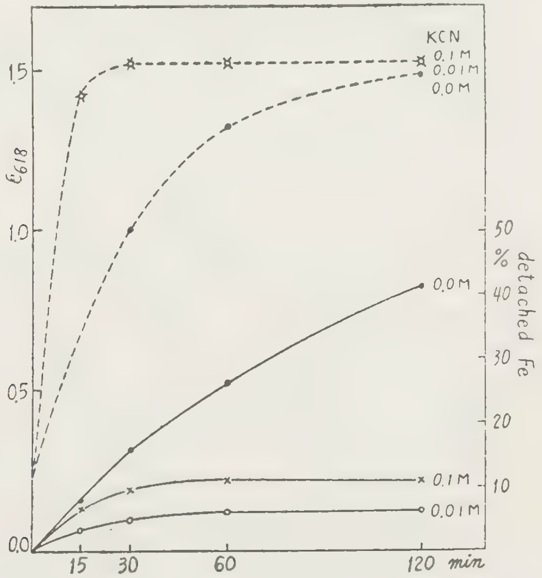

FIG. 4. Formation of pseudohemoglobin and increase of detachable iron content in the reaction of Hb—KCN—ascorbic acid —O_2 system at 38°.

The designations in the figure and the reaction conditions except the temperature are the same as in Fig. 2.

It was also assumed that the nature of the reaction at higher pH caused by K_2CO_3 would be different from that of the ordinary reaction of choleglobin formation. Taking these into considerations, experiments were carried out with varied amounts of K_2CO_3. The results are shown in Figs. 5 and 6. In the earlier stage of the reaction, the reaction rate of the green pigment formation increased with the increase of the concentration of K_2CO_3 added. In the later stage of the reaction, however, the reaction rate fell with the increase of K_2CO_3. With higher K_2CO_3, the reaction was interrupted at the lower yield of the green pigment. Similar relations were observed in the experiment with the detachable iron as shown in Fig. 6. The curves in Fig. 5 and 6, coincided with each other at the lower concentration range of K_2CO_3, while at its higher concentrations two curves were not in parallel; the detachment of iron in the earlier stage of the reaction was much restricted. It may thus be suggested that two different substances are existing in the green pigment produced at higher pH, one of which would hardly liberate its iron and the other more easily, the former being converted into the latter in the course of the reaction process.

At all events, the reaction products at higher pH were proved to be different from the ordinary choleglobin in many points.

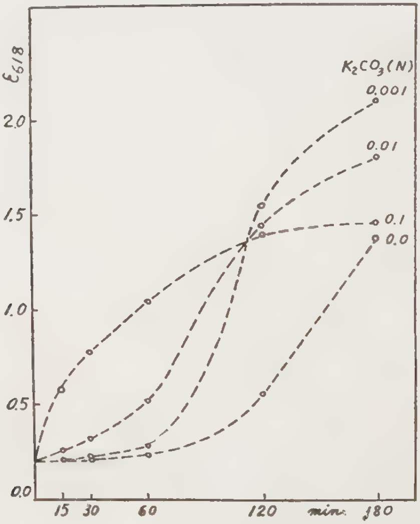

FIG. 5. Green pigment formation in the reaction of Hb—K_2CO_2 —ascorbic acid—O_2 system.

Reaction mixtures: 5.0 ml. of 0.5% HbO_2 (containing catalase), 0.5 ml. of K_2CO_3 in varied concentrations and 0.5 ml. of 0.1 *M* ascorbic acid. T=38°. Designated numbers in the figure represent the final concentrations of K_2CO_3 in *M* (*mole per liter*).

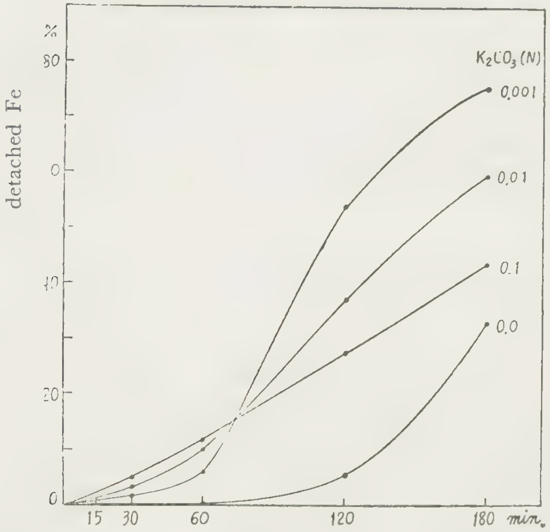

FIG. 6. Detachable iron produced by the reaction of Hb—K_2CO_3—ascorbic acid—O_2 system.

Reaction conditions and the designations in the figure are the same as in Fig. 5.

SUMMARY

Green pigment formation from oxyhemoglobin were studied under varied reaction conditions with special reference to its connection with the easily detachable iron content, detachable by the treatment with 0.4 per cent HCl. The results are summarized as follows:

1. The content of easily detachable iron in the pseudohemoglobin produced by the reaction of Hb—KCN—H_2O_2 system was found to be relatively high. In cases with larger amounts of H_2O_2, it should not be overlooked the possible contamination of a few amounts of iron derived from a sort of by-reaction.

2. The content of easily detachable iron in the pseudohemoglobin produced by the reaction of Hb—KCN—ascorbic acid—O_2 system was very low. The fact suggests the differences of the chemical structure between pseudohemoglobin and choleglobin.

3. The reaction products in the system of Hb—K_2CO_3—ascorbic acid—O_2 at higher pH were assumed to be different from the ordinary choleglobin in many points. At least they do not liberate their iron so

easily as choleglobin.

4. More than two pigments were suggested, further, to be formed in the present reaction system which seemed chemically different from each other. Among them, one would hardly liberate its iron, the other would, however, more easily. The former seemed to be convertible into the latter in the present reaction process. It may be pointed out additionally that a sort of step-reaction seems to take place even in the presence of KCN.

The present investigation was supported by Prof. K. Kaziro, whom the authors wish to thank.

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The Journal of Biochemistry, Vol. 41, No. 2, 1954

ON THE REACTION OF PSEUDOHEMOGLOBIN FORMATION IN THE PRESENCE OF SODIUM DITHIONITE

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(Received for publication, November 2, 1953)

Pseudohemoglobin has hitherto been obtained by the reaction of HbO_2–KCN–H_2O_2 system or of Hb–KCN–ascorbic acid–O_2 system. In the previous paper (1), the pseudohemoglobin formation by these two reaction systems were quantitatively studied. It was evidenced there, that pseudohemoglobin was produced from the ferrous state of hemoglobin and that the ferric hemoglobin produced simultaneously in the reaction process was combined with CN^- to form CN-metHb, so that the latter portion of hemoglobin was excluded from the proper reaction process of pseudohemoglobin formation. The final amount of pseudohemoglobin in the mentioned reaction system was proved to be almost constant irrespectively of the concentrations of ascorbic acid. Thus, hemoglobin was converted in this system into two products, pseudohemoglobin and methemoglobin, and probably in constantly determinated proportion of these two compounds.

From the above facts, it will be expected that the formation of pseudohemoglobin will be increased by keeping the iron in hemoglobin molecule in ferrous state throughout the whole stage of the reaction process. On this expectation, a series of experiments was carried out with the ordinary reaction system of pseudohemoglobin formation under the presence of $Na_2S_2O_2$ added in slight amount. In this system, the reaction of pseudohemoglobin formation proceeded more smoothly and the final yield of pseudoheme from protoheme was found to be much better than that in the ordinary system.

EXPERIMENTAL METHODS

Hemoglobin solution was prepared from the erythrocytes of a patient known as "*Anenzymia catalasea*" (*2*). In some cases in which higher concentration of KCN was added, a hemoglobin solution of rabbit erythrocytes hemolyzate was used. In the latter case, it was proved by the preliminary experiments that catalase in the hemolyzate had been completely inhibited by KCN in experimented concentrations.

Maximum concentration of KCN used was, except in some special cases, as high as 1 *M*. For the dilution of KCN in the reaction mixture, 1 *N* K_2CO_3 was used in order to avoid the change of pH in the reaction solution.

All of the reactions were carried out directly in the absorption cell. Determinations of pseudohemoglobin produced in the system were undertaken by the measurement of its optical density with "Spekker" spectrophotometer.

RESULTS

1. Reaction of Hb–KCN–$Na_2S_2O_4$–Ascorbic–Acid–O_2 System

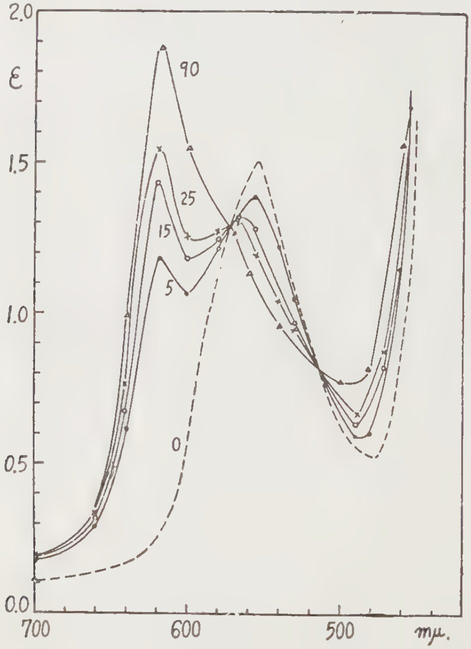

Fig. 1. Changes in the absorption curve followed up with reaction time.

Reaction mixture: 3.0 ml. of 1% HbO_2, 9.0 ml. of *M*/15 phosphate buffer (pH 7.38), 1.5 ml. of 1 *M* KCN, a piece of powdered $Na_2S_2O_4$ and 1.5 ml. of 0.1 *M* ascorbic acid. T=23°. 3 ml. of the reaction mixture was taken into an absorption cell and covered with liquid paraffin after each intervals of reaction as designated in the figure, and then the absorption curve was measured. Dotted line represents the absorption figure of reduced Hb.

Changes of the Absorption Curve throughout the Whole Reaction Process—Oxyhemoglobin solution was taken into an absorption cell, KCN solution and a piece of powdered $Na_2S_2O_4$ were added, but ascorbic acid was not added. The mixture was then allowed to stand in contact with atmospheric oxygen for about 40 minutes. No spectral change occurred in this system. The absorption curve of the reaction solution remained as that of reduced hemoglobin throughout the whole time. When ascorbic acid was added to the reaction solution, however, the reaction solution turned out gradually to green, a distinct absorption maximum being appeared at 618 mμ. Changes of the absorption curve following the reaction time were recorded in Fig. 1. For the measurement of the curves in Fig. 1, the reaction mixture at each interval of time was carefully covered with liquid paraffin in order to cut off the contact of atmospheric oxygen with the reaction mixture. As shown in Fig. 1, the absorption of reduced hemoglobin in the green decreased with time and there arose a new absorption indicating its maximum at 618 mμ which increased as the reaction proceeded. When the globin part was once denatured by the addition of NaOH, the absorption curve in the green turns into that of alkali denatured globin-CN-hemochrome as described in the previous paper. In the red region, however, the absorption maximum was still persisting at 618 mμ. These facts indicated that the green product in the present reaction was also the pseudohemoglobin which has been ordinary obtained in the similar reaction system without the presence of $Na_2S_2O_4$.

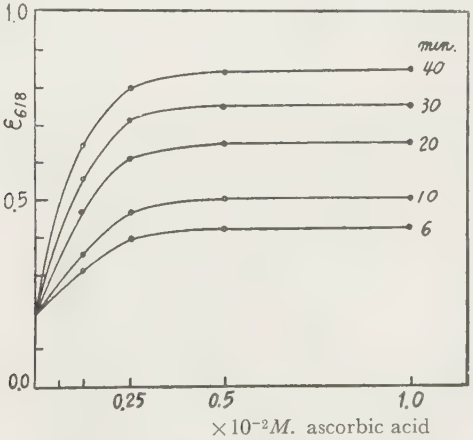

Fig. 2. Effect of the concentration of ascorbic acid.

Reaction mixture: 1 ml. of 1% HbO_2, 3.0 ml. of $M/15$ phosphate buffer (pH 7.17), 0.5 ml. of 1 M KCN, a piece of powdered $Na_2S_2O_4$ and 0.5 ml. of ascorbic acid in varied concentrations. T=25–26°. Designated numbers in the figure represent the times after the reaction started.

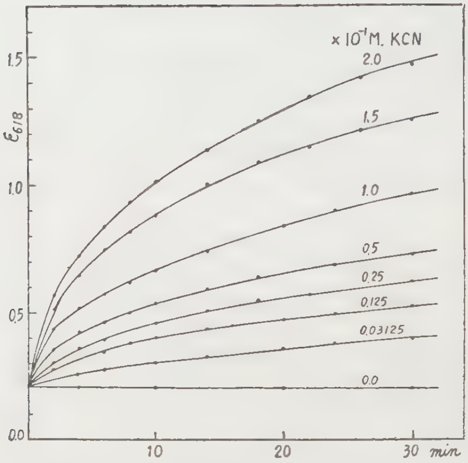

FIG. 3. Effect of the concentration of KCN.

Reaction mixture: 1.0 ml. of 1% HbO_2, 3.0 ml. of $M/15$ phosphate buffer (pH 7.17), 0.5 ml. of KCN in varied concentrations, $Na_2S_2O_4$ and 0.5 ml. of 0.1 M ascorbic acid. T=23.5–24.5°. Designated numbers in the figure represent the final concentrations of KCN in M (mole per liter).

Effect of the Concentration of Ascorbic Acid—When the concentration of hemoglobin and KCN were taken as constant, the reaction rate of pseudohemoglobin formation increased with the increase of the concentration of ascorbic acid added. The maximum rate was reached at final ascorbic acid concentration of $0.5 \times 10^{-2} M$. The amount of $Na_2S_2O_4$ added in this system had no influence upon the reaction rate unless the added amount becomes excessively small. The results are shown in Fig. 2.

Effect of the Concentration of KCN—When the concentrations of hemoglobin and ascorbic acid were taken as constant, the reaction rate increased with increase of the concentration of KCN added. The results are shown in Fig. 3. In the absence of KCN, pseudohemoglobin could never be formed. The plotts of the reaction rate against the concentration of KCN added were given in Fig. 4 and 5. In the lower concentration range of KCN, the plotts were given in hyperbolic and in the higher range of the KCN concentration above 0.025 M, however, rather in a linear relationship. A similar relationship were noticed on the determination of ε_{618} after NaOH was added to the reaction solutions at the end of each interval of time in order to bring the globin part into denaturation as shown in Fig. 6, I.

Experiments with KOCN in Place of KCN—Only KCN has been so far known as effective for the formation of pseudohemoglobin. In the present experiment, however, also KOCN was proved to be effective almost smilarly as KCN. The time curve of the spectral change by KOCN was fairly in accordance with that by KCN. The results are shown in Fig. 6, II. The relationship between the concentration of KOCN and the reaction rate of pseudohemoglobin formation was in good agreement with that founds in the experiment with KCN.

II. Reaction of Hb—KCN—$Na_2S_2O_4$—H_2O_2 System

Much greater amount of pseudohemoglobin was formed when $Na_2S_2O_4$ was preliminarily added to the ordinary reaction system of Hb-KCN-H_2O_2. The absorption figure of the pigment produced in the present reaction system under the presence of $Na_2S_2O_4$ was similar to those shown in Fig. 1.

DISCUSSION

The experimental results so far described seems to indicate doubtlessly that pseudohemoglobin is derived from hemoglobin of ferrous

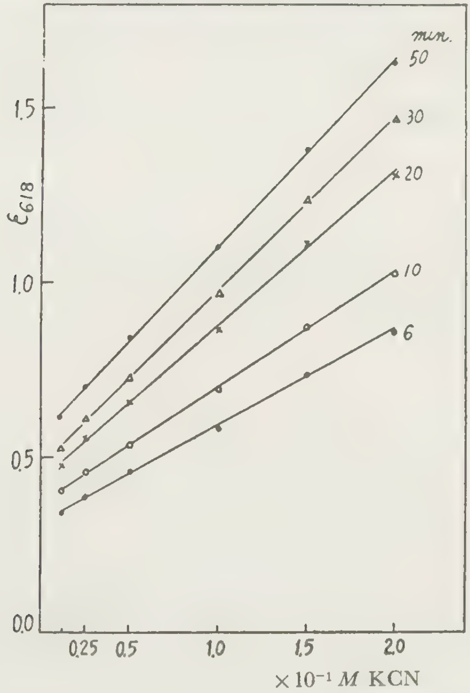

FIG. 4. Relationship between the pseudohemoglobin formation and the concentrations of KCN in the higher range, derived from the experiments shown in Fig. 3.

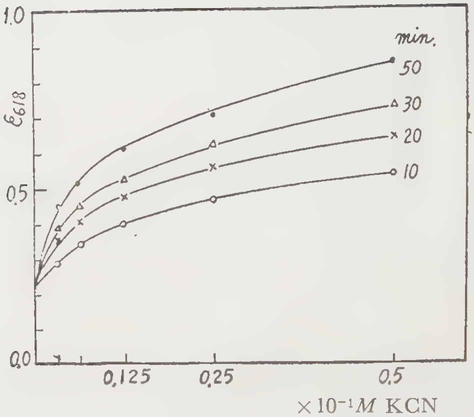

FIG. 5. Relationship between the pseudohemoglobin formation and the concentrations of KCN in the lower range, derived from the experiments shown in Fig. 3.

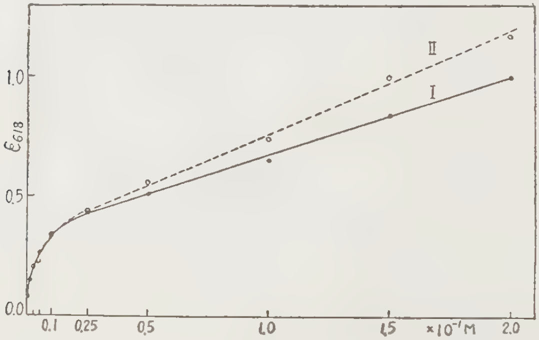

FIG. 6. Relationship between the pseudohemoglobin formation and the concentrations of KCN or KOCN.

Reaction mixtures were made following to that given in Fig. 3. In these series of experiments, ε_{618} was measured after the addition of 0.5 ml. of 6% NaOH to each reaction mixture after the definite time of the reaction. Full line (I): reaction with KCN at 25–25.5°, interrupted by the addition of NaOH at the end of 10 minutes of the reaction. Broken line (II): reactions with KOCN at 8°, interrupted at the end of 15 minutes of the reaction.

state. Since the works of Barkan (*3*), it has hitherto been assumed that hemoglobin can be converted into pseudohemoglobin by the action of H_2O_2 in the presence of KCN. Similar explanation was introduced to the reaction process of pseudohemoglobin formation in the reaction system Hb–KCN-ascorbic acid–O_2 as postulated in the previous paper on the basis of the assumption that H_2O_2 can be generated through the oxidation of ascorbic acid by atmospheric oxygen. The following facts may be pointed out, however, that pseudohemoglobin could not be formed in the reaction system of Hb–KCN–$Na_2S_2O_4$–O_2, although a powerful reductant $Na_2S_2O_4$ was contained in the system and that the production of pseudohemoglobin took place only after the addition of ascorbic acid. And the rate of pseudohemoglobin formation was dependent with the concentration of ascorbic acid added. For all of these, no plausible explanation can be put forward at the present stage of the investigation. It seems, however, that the essential feature of the reaction mechanism of pseudohemoglobin formation can rather be more complicated than has hitherto been acknowledged.

Much remains also to be solved how KCN or KOCN acts in the presented reaction system in favor of the production of pseudohemoglobin from hemoglobin.

SUMMARY

1. Much higher amount of pseudohemoglobin could be formed by the reactions of Hb-KCN–$Na_2S_2O_4$–ascorbic acid–O_2 system and of Hb–KCN–$Na_2S_2O_4$–H_2O_2 system respectively, both of which were introduced by the present authors, than those obtained by the ordinary reactions of Hb–KCN–ascorbic acid-O_2 system or HbO_2-KCN–H_2O_2 system.

2. It may especially be noted that the reaction rate of pseudohemoglobin formation in the reaction of Hb-KCN–$Na_2S_2O_4$–ascorbic acid–O_2 system increased with the increase of the concentration of both ascorbic acid and KCN.

3. KOCN was proved to be samely effective as KCN for the reaction of pseudohemoglobin formation.

4. It may be conclusively stated that pseudohemoglobin is derived from hemoglobin of ferrous state.

5. The mechanism of the reaction of pseudohemoglobin formation seems to be much more complicated than has been explained by Barkan.

The present study was supported by Prof. K. Kaziro, for which the author's deep gratitude is expressed.

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STUDIES ON XANTHURENIC ACID

V. EXPERIMENTS OF PROTRACTED ACCUMULATIVE EFFECTS OF XANTHURENIC ACID CAUSING CHRONIC DIABETIC SYPTOMS IN WHITE RATS

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(Received for publication, November 13, 1953)

In the series of foregoing papers it has been reported that when a fairly large amount of tryptophan was administered at one time to white rats together with sodium salt of fatty acid, following the prescription of Kotake and Inada (*1*), there occurred an abnormal metabolism of tryptophan inducing the formation of xanthurenic acid accompanied with diabetic symptoms.

It had been postulated, therefore, that xanthurenic acid might be an agent responsible for pathogenesis of diabetes mellitus in white rats as well as in man.

But it seems too hasty, to expect the production of such a large amount of xanthurenic acid at one time in ordinary dietetic habits, so it is necessary to examine whether an accumulative effect of small dosis of xanthurenic acid would cause diabetic symptoms. As well known human diabetes is quite chronic. If such an accumulative effect is proved to really occur, this provides a more substantial evidence for the role of xanthurenic acid in the pathogenesis of human diabetes. Using white rats as experimental animal it has been proved that this is really the case. The results will be reported in this paper.

EXPERIMENTAL

(I) To keep the amount of xanthurenic acid constant in their bodies, white rats were fed according to the authors prescription, (*2*) on a diet as shown in Table I containing 35 per cent butter and 25 per cent casein for a long time examined their blood sugar, body weight, amount of xanthurenic acid, and it was found that about 1 mg. of xanthurenic acid was excreted daily in the urine.*

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* The daily output of xanthurenic acid could be increased to 2–3 mg. when the animal had been previously treated for 30 days with the diet, which contained 10 mg. tryptophan.

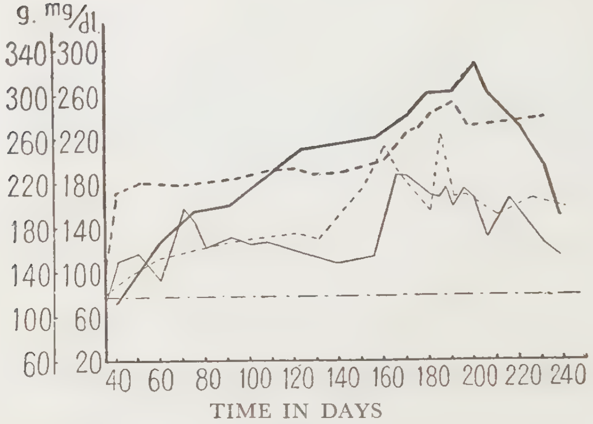


Fig. 1. High fat casein diet

Body weight (g.) Blood sugar (mg./dl.)

As shown in Fig. 1, 2 typical cases are given; the body weight increased regularly, attaining over 300 g. at the end of 200 days and obesity was observed. The blood sugar curve rose gradually in parallel to body weight, the maximum being 180–200 mg./dl. It was estimated by the King and Garner's (*3*) true sugar determining method. After the lapse of 200 days the rats began to lose appetite, body weight decreased rapidly, which the hyperglycemia continued till the animal died finally on about 240th day.

Table I

Casein diet containing rich fat.

Casein	25%	Sugar	5%
McCollum salt	2%	Starch	28%
Agar agar	3%	Medical yeast	2%
Butter	35%		

(II) Next, we adopted another method to keep xanthurenic acid as constant as possible in their bodies. Namely, white rats weighing about 280 g. were fed on a casein diet deficient in vitamin B_6 as shown in Table II. In this way xanthurenic acid could be produced easily in their bodies as pointed out by the research of Lepkovsky *et al.* (*4*). In order to get more enhanced formation of xanthurenic

TABLE II

Casein diet deficient in V. B_6

Hammarsten casein	20%	Salad oil plus liver oil	2%
McCollum salt	4%	Sugar	74%

besides these, following vitamins in each 1 g. food

V. B_1	3.3 γ	Inositol	333.0 γ
Nicotinic acid	10.0 γ	*p*-Aminobenzoic acid	200.0 γ
Calcium pantothenate	13.3 γ	Riboflavin	6.6 γ
Choline chloride	166.0 γ		

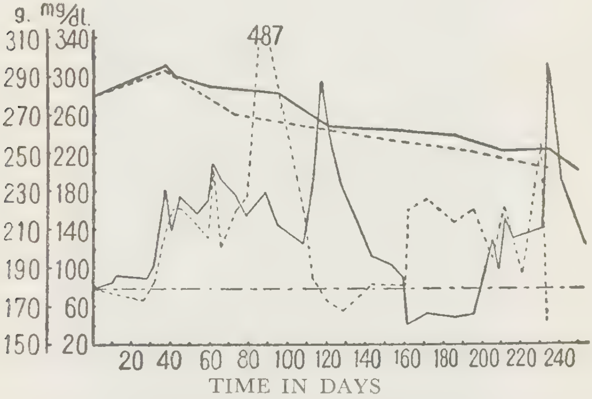


FIG. 2. Casein diet deficient in vitamin B_6

—— - - - Body weight (g.) ...... Blood sugar (mg./dl.)

acid, 10 mg. tryptophan were added after 20th day daily to the diet for 60 days. The daily excreted amount of xanthurenic acid increased to 4–7 mg. on this occasion. The experimental results are illustrated in Fig. 2.

About 1 month after the experiment started, the body weight increased to a slight extent, and the animals died at the end of 8th months. The blood sugar curve began to rise from the 35th day accompaning the symptoms of the vitamin B_6 deficiency, and urinary excretion of xanthurenic acid. Thereafter, the blood sugar was always over 160 mg./dl.; sometimes 300 mg./dl. and even 487 mg./dl. at the highest. On the 159th day, temporary hypoglycemia (40–50 mg./dl.) was seen, but blood sugar rose again to about 200 mg./dl.

(III) On the 190th day of above-mentioned experiment, glucose tolerance was

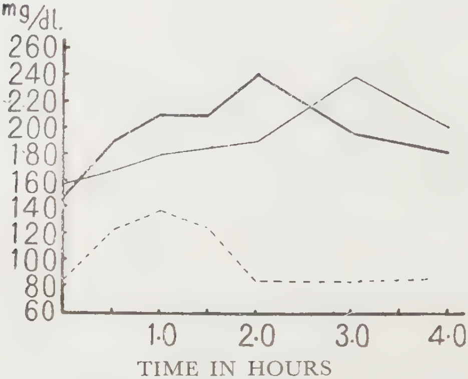


FIG. 3. Glucose tolerance curve glucose 2g./kg. *per os*
——— Casein diet deficient in vitamin B_6, ——— High fat casein diet. - - - - Normal rat

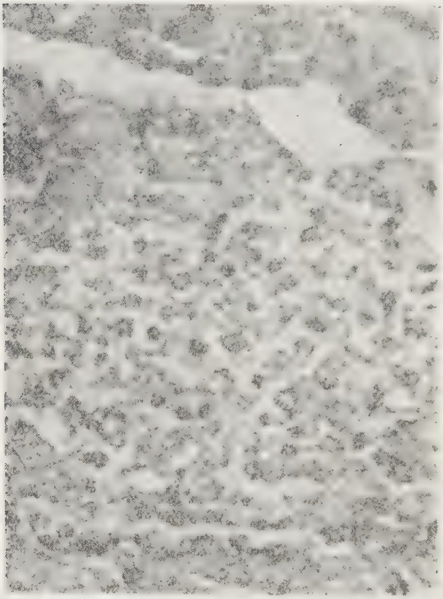
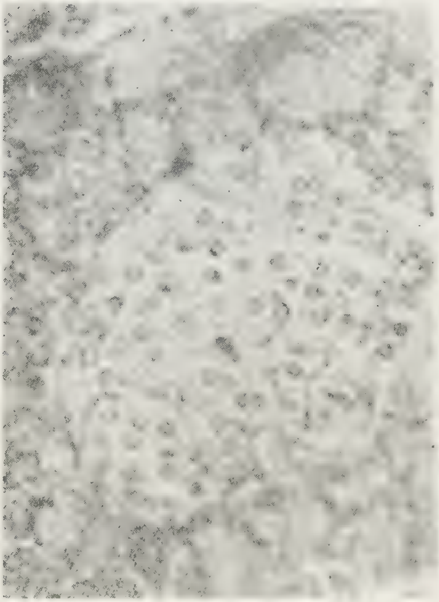


PHOTO. 1. Diet deficient in V. B_6

examined by administering orally 2g. of dextrose per kg. of the body weight. The tolerance dropped remarkably as shown in Fig. 3.

PHOTO. 2. High fat diet



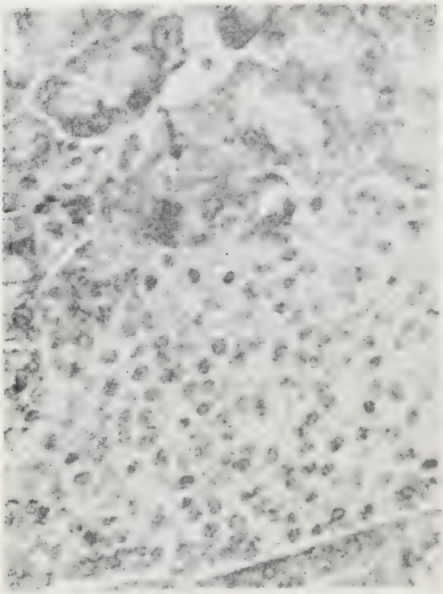
(IV) The animal which showed hyperglycemia on 2 sorts of diets as above mentioned were killed and pancreas was examined microscopically by staining with the Gomori's alumen-hematoxylin-phloxin method (*5*).

In all cases pathological changes of relatively high grade in the Langerhans islets were observed. Photographs show a remarkable drop of stainability, a decrease of the granula, many vacuol formation, an intensive collapse of the protoplasm *etc.* in β-cells of the Langerhans islets, and further some edema of the islets in those white rats fed on a casein diet deficient in vitamin B_6. Changes of the nuclei are not distinct. In the acinus some atrophy of the cells, a decrease of granula and a drop of stainability can be detected. In α-cells of the islets cannot be seen any distinct change. These findings are enough to prove that the diabetic symptoms are truely pancreatic.

CONSIDERATION AND SUMMARY

From the results obtained in the above-mentioned experiments, it is clearly proved that xanthurenic acid is the causative substance of diabetic symptoms in white rats. It is further proved biochemically and histo-pathologically that xanthurenic acid has an accumulative action, namely, when its relatively weak effect continues for certain

PHOTO. 3. Normal



length of time it induces severe chronic diabetic symptoms. The fact that human diabetes generally progresses chronically, is taken into consideration. These findings are of great interest. Further, it should be noted that xanthurenic acid is an abnormal metabolites of tryptophan and that the acid exists always in the urine of diabetes patients as proved by Kotake and Tani (*6*). We, therefore, consider it to be well-founded that the diabetic symptoms caused by xanthurenic acid in white rats is the same with those of human diabetes, caused by nutritional failure. The fact that the existence of a much greater number of diabetes patients among civilized people who eat food rich in fat and protein stands in quite concordance with our view. Indeed, our experimental results would afford a new road for therapy and prophylaxis of human diabetes.

We heartily thank Prof. Yashiro Kotake for his pertinent advices, encouragements and his revision and heartily thank Prof. Keizo Kodama for his revision to this study and also heartily thank the Department of Anatomy and Pathology of our College for their help given us in the histopathological examinations.

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STUDIES ON XANTHURENIC ACID

VI. EFFECT OF XANTHURENIC ACID UPON GLYCOGEN CONTENTS IN LIVER, HEART MUSCLE AND SKELETAL MUSCLE

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(Received for publication, November 13, 1953)

In 1935, Imagawa (*1*) reported that the glycogen contents in liver and skeletal muscle decreased in a depancreastized dog as well as in a human body whose death was caused by diabetes. This finding was confirmed by Mitsunaga in 1942 (*2*). But the results obtained by a number of investigators on animals of alloxan diabetes are divergent. Lackey *et al.* (*3*) found that glycogen in the heart muscle increased, while that in the skeletal muscle and liver decreased. Miller (*4*) reported to the contrary that glycogen in the liver increased. Similar experiments were made by Shimazono (*5*) using rabbits and white rats as experimental animals, a decrease of glycogen in all those three tissues was confirmed. Further, in 1951, alloxan-injected rabbits were examined by Numaguchi (*6*), and the results obtained were reported as follows. More detailed study in the same line by Numaguchi with alloxan diabetic rabbit gave somewhat different results. Namely, at primary hyperglycemia period the amount of glycogen in liver and skeletal muscle decreased, while that in heart muscle suffered little or no change. At hypoglycemia period glycogen in liver and heart muscle increased somewhat than at primary hyperglycemia period, but in skeletal muscle it continued to decrease. At second hyperglycemia period glycogen in liver and skeletal muscle showed a pronounced decrease, while that in heart muscle rather a remarkable increase.

In the cases of our experimental xanthurenic acid diabetes (*7*) a remarkable rise and fall of the glycogen amount was observed which proceeded in a similar way to cases of alloxan diabetes. This fact indicates not only an important effect of xanthurenic acid on glucose metabolism, but also a further conclusive evidence for the occurrence of diabetic symptoms in white rats by xanthurenic acid.

EXPERIMENTS

Materials and Method—Experiments were repeatedly made on male white rats, about 150 g. in weight, and fed on a diet as shown in the following table for one week. The last meal was given at 3 p.m. and next day at *a.m.* xanthurenic acid (200 mg. per kg. body weight) was injected into the peritoneal cavity. Then at the lapse of 4, 6–8 and 72 hours after injection, which correspond to the primary hyperglycemic, the hypoglycemic and the second hyperglycemic period respectively the animals were brought to death and treated as follows.

Synthetic diet

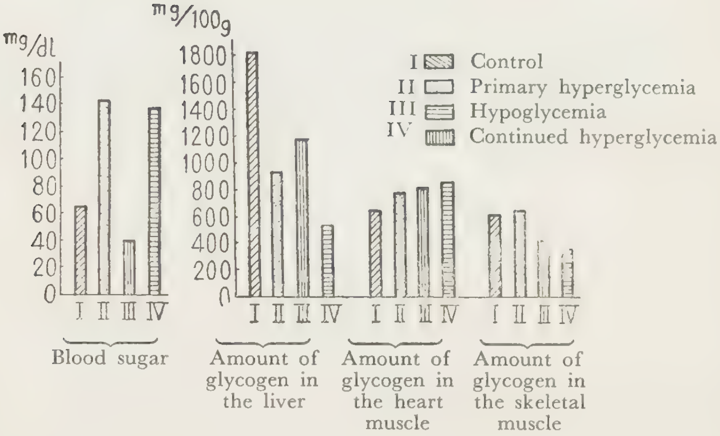
Casein	22 %	McCollum salt	6 %
Agar agar	3 %	Yeast	2 %
Butter	10 %	Sugar	5 %
Starch	52 %		

The carotid artery on both sides was cut and the blood let out thoroughly; then the abdominal and chest cavities were opened immediately, and the organ slices were prepared out of the marginal part of the left lobe of the liver and similarly out of the left ventricle of the heart. These slices and a part of the right sternal vastus muscle were used as test materials.

The glycogen measurement was made by the Kurokawa's (*8*) method. First a test-material, about 20 mg. in weight, was made free from blood by sucking off with filter papers, weighed immediately by means of a tortion balance and dipped into a test-tube, containing 0.5 ml. of previously warmed 30 per cent KOH solution. After tightly stoppered, it was warmed for 1 hour in a hot water bath, and then cooled. Further, 2.0 ml. of absolute alcohol and 0.1 ml. of 1 per cent Na_2SO_4 solution were added to it, the whole content was shaken well, and kept standing over night. In this way glycogen came out as sediment.

After the upper clear portion was centrifuged off the sediment was washed, treated with each 2.0 ml. of 70 per cent, then 95 per cent alcohol, and lastly with petroleum ether, dried at 100°, mixed with 1 ml. of 5 per cent HCl, warmed for 1 hour in a boiling water-bath to convert glycogen into glucose. This solution was neutralized with 1.5–3 per cent NaOH using phenolphtalein as an indicator. Then 5 ml. of 0.45 per cent $ZnSO_4$ and 1 ml. of $N/10$ NaOH were added to and protein was removed. After that, the glucose amount was measured by the Hagedorn-Jansen's method, and the glycogen amount was calculated by glucose amount × 1.09. The results are illustrated in the figure, where the mean value on 5 rats are given.

As illustrated, in the primary hyperglycemia period, glycogen in the liver decreased to half of the control value and in the second hyperglycemia period to less than 1/3. But glycogen in the heart muscle rather showed an increasing tendency in the second hyperglycemia period, while in skeletal muscle it showed a decrease less than half of



control value. These results agree in general with those obtained by Numaguchi and others on alloxan diabets animals.

SUMMARY

We were able to observe remarkable change in the glycogen content in liver, heart muscle and skeletal muscle of the xanthurenic-acid-injected white rats. This affords an evidence for a pronounced effect of xanthurenic acid on glucose metabolism which stands in accord to diabetic symptoms induced by xanthurenic acid.

In conclusion, we heartily thank Prof. Yashiro Kotake for his pertinent advices, encouragements and his revision and also heartily thank Prof. Keizo Kodama for his revision to this study.

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OXIDATION OF 7-KETO BILE ACIDS WITH SELENIUM DIOXIDE. I

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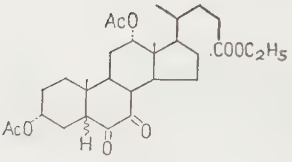
(Received for publication, November 15, 1953)

According to Louis F. Fieser *et al.* (*1*) dehydrogenation of methyl 3,7-diacetoxy-12-ketocholonate by selenium dioxide in acetic acid produced methyl 3,7-diacetoxy-12-keto-$\Delta^{9,(11)}$-cholenate which was convertible to 3-hydroxy-12-keto-$\Delta^{7,9,(11)}$-choladienic acid by excess of aqueous methanolic alkali. In this paper the results of the oxidation of ethyl 3,12-diacetoxy-7-ketocholanate (*2*) with selenium dioxide are described. It was remarkable that the oxidation product was different according to the conditions of reaction, especially the reaction temperature.

The oxidation on a water bath gave two crystalline compounds (A) with m.p. 180° and (B) with m.p. 153°. Under the same conditions two cases occurred; in the one case, only (A) was obtained and in the other case, both (A) and (B) a little (A) and much (B) were isolated. The reason of different yield is unknown.

(A) corresponds to the formula $C_{30}H_{44}O_8$ and gives green color with $FeCl_3$. The maximum of its ultraviolet absorption spectrum is $\lambda_{\text{max}}^{\text{dioxane}}$ 277 mμ (logε 4.05). Its structure may be assumed to be (I) from the view-point that (A) may be the α-diketo compound. But since it does not react with *o*-phenylenediamine. It seems to have the structure of enol form (II) in the solution. (B) corresponds to the formula $C_{25}H_{40}O_6$, is positive for tetranitromethane, and gives violet color with $FeCl_3$. The maximum of its ultraviolet absorption spectrum is $\lambda_{\text{max.}}^{\text{dioxane}}$ 311 mμ (logε 4.3). From its color reaction (B) is expected to have α-diketone group and one double bond. Its formula further corresponds to that which lost one molecule of acetic acid from (A). From these results it may also be assumed that (B) would have the structure of (III) (*3*, *4*, *5*).

The non-crystallized fractions which (A) and (B) were separated, were again oxidized with selenium dioxide in a mixture of glacial acetic



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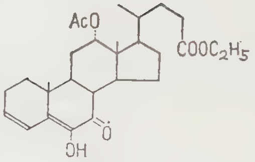
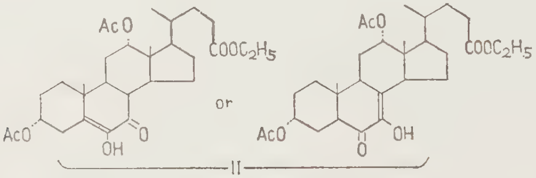


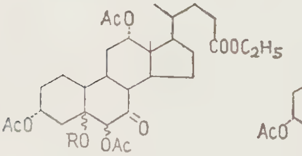
III

acid and acetic anhydride on a water bath for 5 hours. The products could not be crystallized. By further treatment of the products with pyridine and acetic anhydride were the crystals obtained which began to sinter at about 100° and melted at 115° with bubbling. $FeCl_3$ color reaction is negative. The crystal form, solubility and melting point are identical with those of the next crystal (C).

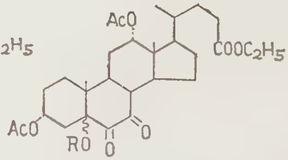
Next, the oxidation on an oil bath at 120° afforded a needle-like crystal (C) with m.p. 108–112° (bubbling), which did not show the clear melting point even on recrystallization from various solvents. Tetranitromethane, $FeCl_3$ color reaction and Tortelli-Jaffe test are all negative. Its analysis corresponds to $C_{33}H_{48}O_{11}$ or $C_{34}H_{50}O_{11}$ and the maxima of the ultraviolet absorption spectrum are 288 mμ (logε 1.85) and 375 mμ (logε 1.62). In the view of its analysis (V) or (VII) may be supposed to be the structure of (C), but as its $\lambda_{\text{max.}}^{\text{dioxane}}$ and logε correspond to those of the known α-diketo steroids, its structure is consequently assumed to be (VII) rather than (V). In this case neither (A) nor (B) was isolated.



II



IV R = H
V R = Ac



VI R = H
VII R = Ac

Thus, the structures of (A), (B) and (C) produced by the action of selenium dioxide on ethyl 3,12-diacetoxy-7-ketocholanate were respectively presumed. The confirmation will be later reported.

The author takes this opportunity to extend his deep gratitude to Prof. T. Kazuno who has given him kind guidance during this work. The author extends his thanks to Messrs. T. Iki, and K. Miyahara for carring out elementary analysis, and to Messrs. T. Kubota, K. Tori and Y. Matsui for taking charge of absorption spectra.

EXPERIMENTAL

All melting points are uncorrected.

I. The Reaction on a Water Bath

(i) Ten g. of ethyl 3,12-dihydroxy-7-ketocholanate were acetylated to its diacetate with a mixture of 40 ml. of acetic anhydride and 40 ml. of glacial acetic acid on a water bath for 5 hours. To the reaction mixture were added then 5 g. of selenium dioxide and 40 ml. of glacial acetic acid, and the heating was continued for further 10 hours. After cooling, the solution was filtered and the filtrate was evaporated to dryness under reduced pressure. The residue was extracted with ether and the ether solution was washed with water, 2 per cent sodium carbonate solution and water, dried over sodium sulfate and the ether was evaporated. The non-crystallized residue was dissolved in 60 ml. of benzene-petroleum ether (1 : 2) and chromatographed over 100 g. of alumina. Elution was performed with benzene-petroleum ether (1 : 2, 1 : 1 and 2 : 1 ; each 300 ml.), benzene (300 ml.) and then benzene-ethanol (9 :1, 3 :1, 1 :1 ; each 200 ml.). After removal of the solvents from the combined benzene-petroleum ether eluates, the residue was crystallized from methanol. Recrystallization from ethanol gave crystals with constant m.p. 178.5–180°, which showed a deep green color with $FeCl_3$, $\lambda_{max.}^{dioxane}$ 277 mμ (logε 4.05).

Analysis: Calcd. for $C_{30}H_{44}O_8$: C, 67.67 ; H, 8.27.
Found: C, 67.31 ; H, 7.91.

From the benzene eluate and benzene-ethanol eluate no crystal was obtained.

(ii) A solution of 10 g. of ethyl 3,12-dihydroxy-7-ketocholanate in a mixture of 20 ml. of pyridine and 20 ml. of acetic anhydride was allowed to stand overnight at room temperature. From the reaction solution the solvents were evaporated to dryness under reduced pressure and the residue was dissolved in ether. The ether solution was washed with water, dilute hydrochloric acid, water, 2 per cent sodium carbonate solution and water, dried over sodium sulfate. After evaporating the solvent the residual ethyl 3,12-diacetoxy-7-ketocholanate was dissolved in a mixture of 80 ml. of glacial acetic acid and 40 ml. of acetic anhydride. To this solution were added 5 g. of selenium dioxide and the mixture was warmed on a water bath for 8 hours. After the completed reaction the solution was treated in the same way as above. The product was dissolved in benzene-petroleum ether (1 : 3) and chromatographed and eluted with benzene-petroleum ether (1 : 3, 1 : 1 and 3 : 1 ; each 200 ml.), benzene (200 ml.), benzene-ether (3 : 1 and 1 : 1) and then benzene-ethanol (3 : 1 and 1 : 1). The benzene-petroleum ether eluates were combined and the solvent was evaporated. The residue was recrystallized from methanol to give crystals with m.p. 175–180°. This gave green color with $FeCl_3$. The melting point was not depressed by admixture with the above crystal of m.p. 178.5–180°. No crystal was obtained from benzene eluates. The benzene-ether and benzene-ethanol eluates were combined and the solvents evaporated. The residue was dissolved in benzene-petroleum ether (1 : 1), passed through an alumina column, and recrystallization from ethanol gave colorless prisms with m.p. 152–153°. It was positive for tetranitromethane and gave violet color with $FeCl_3$.

Analysis: Calcd. for $C_{28}H_{40}O_6$: C, 71.19; H, 8.47.
Found: C, 70.79; H, 8.21.

Ultraviolet absorption spectrum; $\lambda_{\text{max.}}^{\text{dioxane}}$ 311 mμ (log ε 4.3).

(iii) Fxcept the fraction from which crystals of (A) and (B) were separated, all non-crystallized fractions on the above chromatography were combined and dissolved in a mixture of 40 ml. of acetic anhydride and 80 ml. of glacial acetic acid. To this solution were added 5 g. of selenium dioxide and the mixture was warmed on a water bath for 15 hours. After the completed reaction the mixture was treated as mentioned above and the resulting non-crystallized product was dissolved in pyridine and acetic anhydride, and warmed on a water bath for 5 hours. After treating in the usual method, about 0.3 g. of needle-like crystals were obtained from benzene-petroleum ether eluates. The crystal sintered at 100–110° and melted at 115° with bubbling. It is negative for $FeCl_3$. These crystals fully resemble to the next crystal (C) in form, melting point and solubility.

II. The Reaction on an Oil Bath

After 10 g. of ethyl 3,12-dihydroxy-7-ketocholanate were acetylated by the same method as above, it was dissolved in a mixture of 80 ml. glacial acetic acid and 40 ml. of acetic anhydride and to the solution was added 5 g. of selenium dioxide. The mixture was gently refluxed in an oil bath for 5 hours at 115–120°. After cooling, it was treated as usual. The product was recrystallized from benzene to give about 1 g. of needles with m.p. 180–110° (bubbling). Though it was repeatedly recrystallized from ethyl acetate-petroleum ether or ethanol, it did not show a clear melting point. It is negative for tetranitromethane, $FeCl_3$ and Toretelli-Jaffe test

Analysis: Calcd. for $C_{32}H_{46}O_{10}\cdot\frac{1}{2}H_2O$: C, 64.11; H, 7.85.
,, $\cdot\frac{1}{2}CH_3COOH$: C, 63.87; H, 7.74.
$C_{34}H_{50}O_{11}$: C, 64.35; H, 7.89.
Found: C, 63.69, 63.67; H, 7.86, 8.04.

Ultraviolet absorption spectrum; $\lambda_{\text{max.}}^{\text{dioxane}}$ 288 mμ (log ε 1.85), 375 mμ (log ε 1.62).

SUMMARY

The oxidation of ethyl 3,12-diacetoxy-7-ketocholanate with selenium dioxide afforded three products and these structure were presumed.

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The Journal of Biochemistry, Vol. 41, No. 2, 1954

"ALLITHIAMINE" A NEWLY FOUND DERIVATIVE OF VITAMIN B_1

II. THE EFFECT OF ALLITHIAMINE ON LIVING ORGANISM

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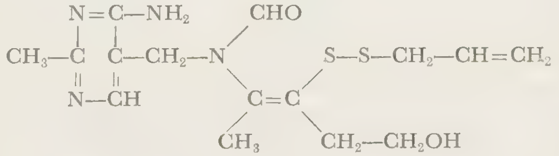
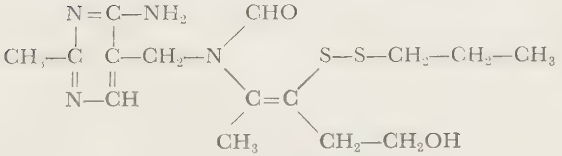
(Received for publication, November 24, 1953)

The previous report (*1*) has dealt with the discovery of allithiamine, a newly found derivative of vitamin B_1, which was formed from thiamine by mixing it with the extract of garlic (*Allium sativum*). The present report is intended to present the results of our study on the effects of allithiamine and its homologue on living organism as compared with that of thiamine.

Allithiamine and its homologue used in this study have been prepared synthetically by Dr. T. Matsukawa *et al.*, our cooperators (*2*, *3*). For the sake of simplicity allithiamine and its homologue will be represented by TAD and TPD respectively in the present report.



TAD



TPD

Toxicity Test

Intravenous or oral LD 50 has been determined for a series of mice weighing 10 to 15 gm. by giving them thiamine, TAD and TPD intravenously or orally according to routine method. The results are shown in Tables I and II.

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TABLE I

The Intravenous LD 50 for Mice

(Milligram per 10 grams of body weight)

	ATD	TPD	Thiamine
	mg.	mg.	mg.
LD 50	3.40	3.52	1.11
Upper border of LD 50	3.85	3.78	1.21
Lower border of LD 50	3.01	3.27	1.02

pH of the solution of injection was 6.8. LD 50 was calculated by probit transformation in customarily manner.

TABLE II

The Oral LD 50 for Mice

(Milligram per 10 grams of body weight)

	TAD	TPD	Thiamine
	mg.	mg.	mg.
LD 50	8.50	15.80	>30
Upper border of LD 50	10.06	18.80	—
Lower border of LD 50	7.18	13.28	—

While intravenous LD 50 of thiamine-HCl was 1.11 mg., that of TAD and TPD was found to be 3.40 and 3.52 mg. respectively, suggesting the fact that the latter two compounds are less toxic than thiamine-HCl. In contrast with this, oral LD 50 test yielded an interesting result; namely, while a large dose of thiamine-HCl exceeding 30 mg. did not result fatally, that of TAD and TPD killed animals. This discrepancy in the results of the two tests is interpreted to show that thiamine is resistant to a rapid absorption from the intestine.

Growth Test in Rats

Young rats, kept upon thiamine deficient diet, were observed for their responce in growth to oral administration of a certain dose of thiamine, TAD and TPD. The results are shown below.

I. The First Experiment—A growth test has been run on 4 groups of rats, with 5 animals in each group, in the course over one month, one group receiving no thiamine (B_1-deficient group), one group 40 γ of thiamine-HCl, one group 42 γ of TAD and one group the same dose of TPD (equivalent of 4 0γ of thiamine-HCl in mole) daily. The results are shown in Table III.

TABLE III

Prevention of V.B$_1$ Deficiency in Rats by Thiamine, TAD and TPD

Treatment	Average weight					
	Initial	1 wk.	2 wks.	3 wks.	4 wks.	5 wks.
	g.	g.	g.	g.	g.	g.
Basal deficient diet	42	53	62	52	all dead	
B$_1$-HCl (40γ) addition	44	60	74	87	96	118
TAD (42γ) addition	42	55	76	93	110	127
TPD (42γ) addition	41	57	72	90	115	132

Basal deficient diet used in rat experiments: starch 10.2 g., casein 2.7 g., salts 0.6 g., soy-bean oil 0.8 g., cod-liver oil 0.3 g., riboflavin 20γ, V.B$_6$ 25γ, nicotineamide 100γ, Ca pantothenate 100γ per head daily.

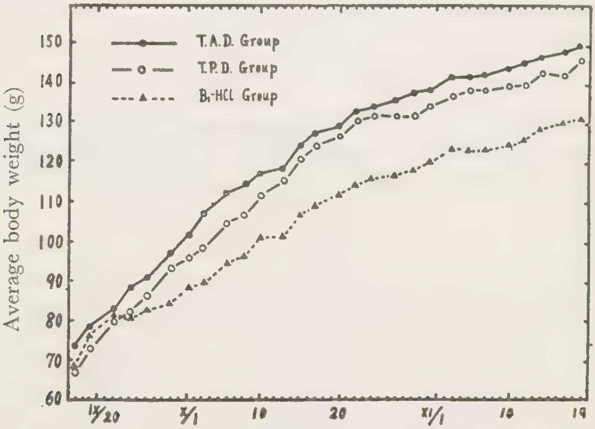

FIG. 1. Growth curve in rats. Receiving doses: B$_1$-HCl group, 40γ: TAD and TPD group, 42γ, equimolar dose of thiamine, daily.

II. The Second Experiment—Similar test has beed run on a bunch of litters of rat. The results are shown in Fig. 1.

III. The Third Experiment—Growth test has been run using 1.50γ of thiamine-HCl and 1.75γ of TPD, with five rats in each group. The results are presented in Fig. 2.

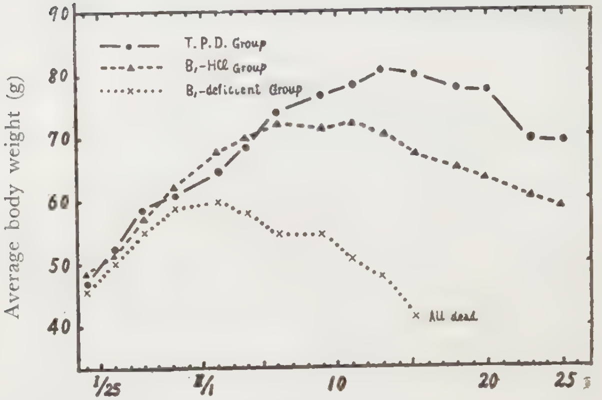

Fig. 2. Growth curve in rats. Receiving doses: B_1-HCl group, 1.50 γ; TPD group, 1.75γ, equimolar dose of thiamine, daily.

As reviewed statistically the difference was found to be highly significant between TAD or TPD group and thiamine group so far as the first and second experiments were concerned.

Bradycardia Test in Rats

Rats have been kept upon thiamine deficient diet as above, until their pulse rates fell to 250 to 300, whereupon they were given 10γ of thiamine or 10.5γ of TAD or TPD and observed for the duration of cures of their pulse rate. The results are shown in Table IV.

Table IV

Duration of Cures in Heart Rate after Administration of Thiamine, TAD and TPD

Thiamine	TAD	TPD
days	*days*	*days*
3	4	4
5	4	5
3	5	4
2	5	3
3	4	4
4	4	4
4	3	5

Amount of oral doses were 10γ of thiamine and 10.5γ of TAD and TPD equimolar dose of thiamine.

No appreciable difference was observed among the curative effects of thiamine, TAD and TPD on bradycardia. This held also for their curative effects on convulsion of thiamine deficient pigeon.

Excretion of Thiamine from Human Subjects who Received Allithiamine Orally

We are familiar with the fact that, when a large dose of thiamine is ingested orally, most part of it is readily excreted into feces, owing to its poor permeation through intestinal wall. In contrast with this, allithiamine is very easily absorbed from the intestine. The following experiment was intended to compare allithiamine with thiamine in detail in term of their absorption from the intestine.

Six subjects who are healthy male university students had been on the same diet for a week preceding the study. Thereafter thiamine, TAD and TPD has been given to them, as divided into three groups of each 2 individuals, at each diet in daily dose of 2 mg. in the first week, in increasing doses thereafter as 5 mg., 10 mg. and 20 mg., and their urinary and fecal contents of thiamine were estimated. The results are shown below.

No excretion of TAD or TPD as it is, has been found in every individuals receiving TAD or TPD. Figs. 3 and 4 present the urinary excretion of thiamine as observed in each one individual from thiamine and TPD groups.

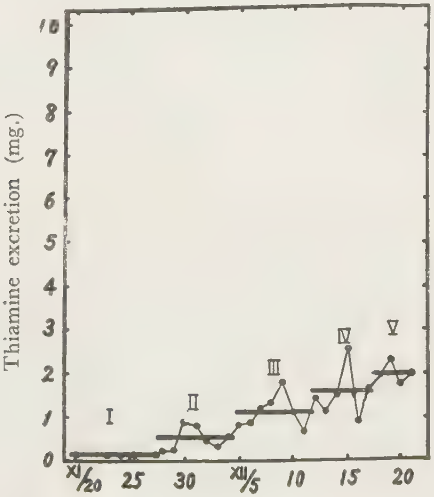

FIG. 3. Urinary excretion of thiamine in a subject (In) receiving thiamine. Thick line shows the average level of thiamine excretion in each experimental period. I: control period, II: 2 mg. fed period, III: 5 mg. fed period, IV: 10 mg. fed period, V: 20 mg. fed period.

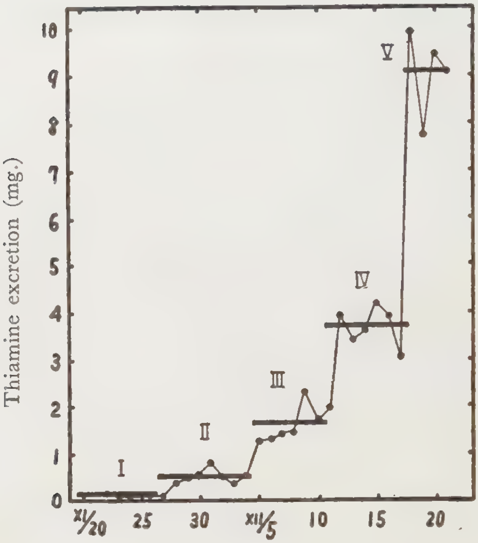

FIG. 4. Urinary excretion of thiamine in a subject (H.) receiving TPD.

Thick line shows the average level of thiamine excretion in each experimental period. I: control period, II: 2 mg. fed period, III: 5 mg. fed period, IV: 10 mg. fed period, V: 20 mg. fed period.

The fecal levels of thiamine as observed in the same subjects are shown in Figs. 5 and 6.

The average levels of excretion in all subjects at different time of feeding are shown in Tables V and VI.

While the urinary excretion of thiamine increased in TAD and TPD group linearly with the increase in intake, no equally distinct increase occurred in urinary discharge of thiamine in thiamine group. Although thiamine ingestion in dose of 2 mg. caused an only slite increase in fecal level of thiamine, that of 20 mg. caused an abrupt and tremendous increase. On the other hand 20 mg. of TAD or TPD were responsible for a fairly abrupt increase in the fecal discharge of thiamine, but the increase was not comparable in magnitude with the one caused by 20 mg. of thiamine.

Effect of Allithiamine Administration on Blood Level of Thiamine

I. Experiment in Rabbits—After the intravenous injection of thiamine and that of

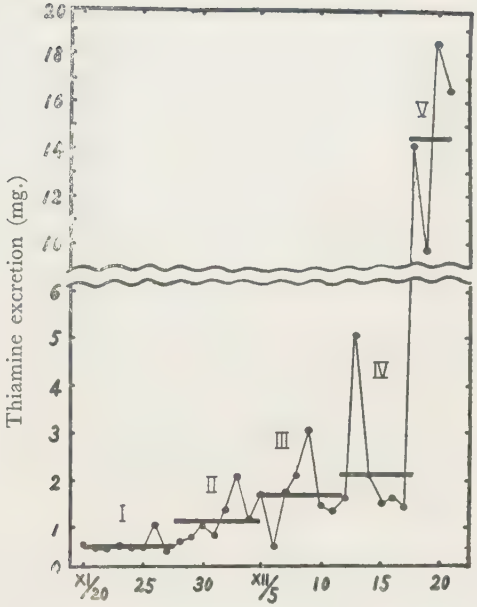

FIG. 5. Fecal excretion of thiamine in a subject (In.) receiving thiamine.

Thick line shows the average level of thiamine excretion in each experimental period. I: control period. II: 2 mg. fed period, III: 5 mg. fed period, IV: 10 mg. fed period, V: 20 mg. fed period.

TPD in dose of 10 mg. and 10.6 mg. respectively, the blood level of total thiamine as well as the thiamine content in blood cells and in plasm have been determined in rabbits at different points of time. The results are shown in Figs. 7 and 8.

As to the blood level of total thiamine, the patterns of response simulated one another among thiamine and TPD. There was a rise immediately after the injection, followed by a gradual decline, and thiamine above all induced a typical pattern of such type. However, a marked difference was shown to exist among them in magnitude; namely, blood level of total thiamine was found to be elevated by *ca.* 600 γ/dl. immediately following the injection of thiamine and reduced to *ca.* 100 γ/dl. after one hour, whilst the initial elevation after TPD injection reached as high value as *ca.* 2,800 γ/dl. with sustained effect even after one hour when the value was still as high as *ca.* 1,000 γ/dl.

As to the thiamine concentrations in the blood cell and plasm, thiamine and TPD were shown to behave in different ways; namely, while thiamine injection was res-

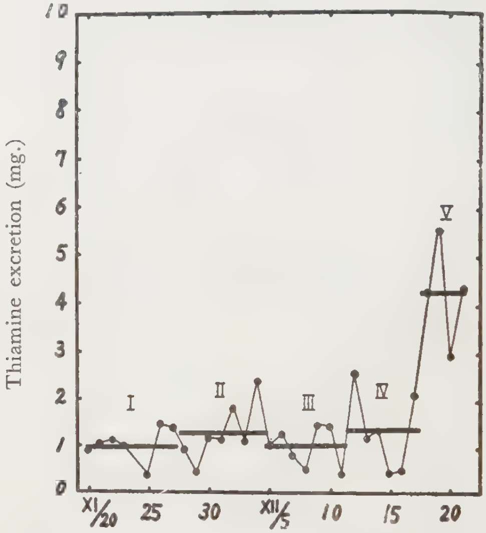

FIG. 6. Fecal excretion of thiamine in a subject (H.) receiving TPD. Thick line shows the average level of thiamine excretion in each experimental period. I: control period, II: 2 mg. fed period, III: 5 mg. fed period, IV: 10 mg. fed period, V: 20 mg. fed period.

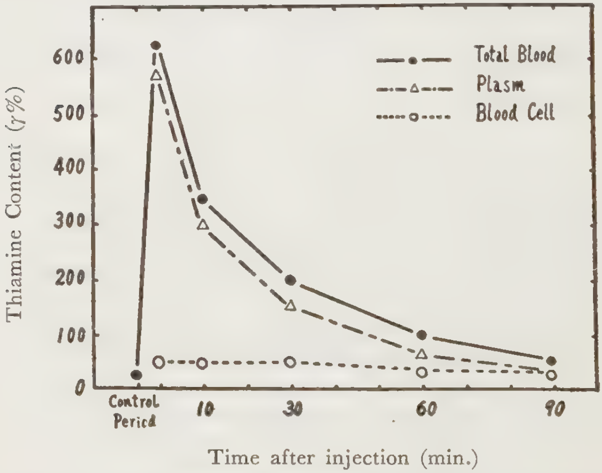

FIG. 7. The increase of blood level of thiamine after intravenous injection of thiamine (rabbit). Dose: 10 mg.

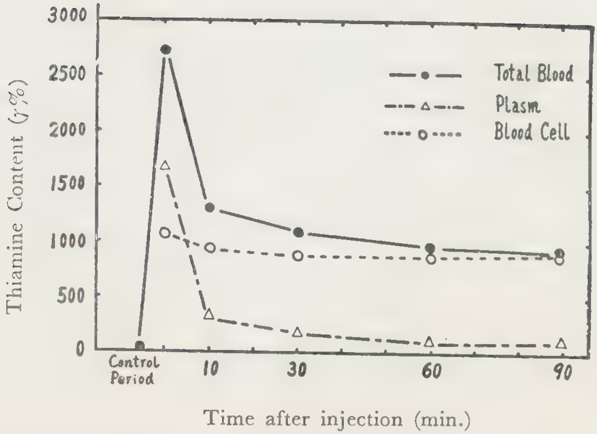

FIG. 8. The increase of blood level of thiamine after intravenous injection of TPD. (rabbit). Dose: 10.6 mg.

TABLE V

Average Daily Urinary Excretion of Thiamine in Response to Administration of Different Doses of Substance

		Thiamine group		TAD group		TPD group	
Subject		I.	In	S.	E.	O.	H.
Control period		145^{γ}	70^{γ}	85^{γ}	88^{γ}	92^{γ}	90^{γ}
Experimental period	2 *mg.*	673	516	542	662	674	544
	5 *mg.*	1418	1093	1609	1791	1259	1682
	10 *mg.*	1022	1554	3649	4332	3776	3708
	20 *mg.*	2094	1990	6300	6617	7879	9043

ponsible for a poorer rise of thiamine concentration in the blood cell than in plasm, TPD injection was found responsible for an outstanding rise of thiamine concentration *i.e.*, more rapid and greater in magnitude and yet more persistent, in the blood cells than in plasm.

II. Experiment in Human Being—Fig. 9 illustrates the response of blood level of thiamine to 20 mg. of injection of TAD and thiamine. The results are consistent with the ones obtained in preceding experiment on rabbits; namely, after TAD injection the blood level of thiamine was sustained at a highly elevated level for a long period of time.

TABLE VI

Average Daily Fecal Excretion of Thiamine in Response to Administration of Different Doses of Substance

		Thiamine group		TAD group		TPD group	
Subject		I.	In.	S.	E.	O.	H.
Control period		682γ	664γ	785γ	782γ	1130γ	1020γ
Experimental period	2 *mg.*	1387	1159	756	992	1104	1319
	5 *mg.*	1493	1716	685	864	736	1040
	10 *mg.*	1354	2230	1278	1162	1367	1345
	20 *mg.*	12318	14510	3210	6335	2369	4196

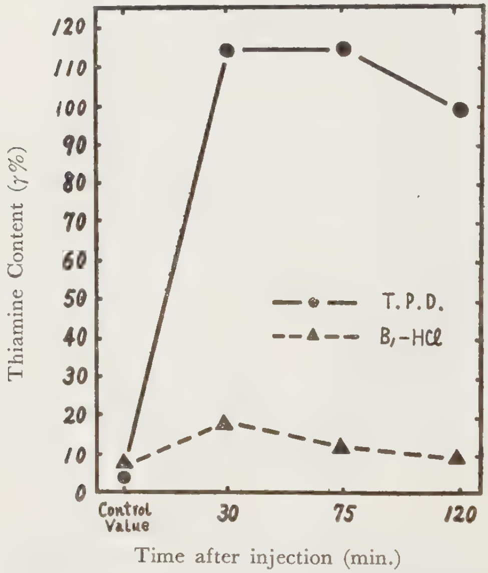

FIG. 9. The increase of blood level of thiamine after subcutaneous injection in dose of 20 mg. into a subject 20 years of age.

Thiamine Concentration in the Organs of Animals Receiving Allithiamine Orally

It has been demonstrated in the preceding chapter that the urinary excretion of thiamine increased after the oral administration of TAD to a greater extent than after the ingestion of the equivalent dose of thiamine. A further study was made on the thiamine concentrations in the organs of animals which received TAD or thiamine while alive. A bunch of litters of rats, placed on the paired sets of different conditions for a certain period of time preceding the study, has been given the same does of thiamine or TAD orally, and sacrificed after 2 hours. Table VII presents the thiamine concentrations in various organs.

TABLE VII

Thiamine Content in Organs of Animals after Oral Administration of Thiamine or TAD

	Number of paired set of animals.	Thiamine administration	TAD administration
		%	%
Liver	1	495	1228
	2	255	460
	3	332	756
	5	425	882
	7	604	1055
Kidney	2	141	195
	3	125	251
Stomach	6	100	128
	7	153	239
Small Intestine	1	84	236
	4	100	179
Heart	3	76	96
	4	109	107
Lung	4	113	108
	6	64	93
Spleen	6	130	154
Brain	2	206	207
	5	199	205
Blood	6	18	36
	7	27	32

Amounts of thiamine and allithiamine used in this experiment were 50γ and 52.5γ, respectively.

Most organs from an animal receiving TAD while alive were found to be richer in thiamine content than their equivalents from the one reciving thiamine.

Phosphorylation of Allithiamine in Living Body

After feeding animals in the same way as in the foregoing experiment, we gave them either TAD or thiamine, sacrificed them and examined their livers for the content of total thiamine and free thiamine. Table VIII shows the amounts of free thiamine in ratio to total thiamine.

TABLE VIII

The Ratio of Free Thiamine Content to the Total Thiamine Content in the Liver

Method of administration	Thiamine administration	TAD administration
	%	%
Oral administration of 40 γ daily for 4 days, sacrificed afterward.	7.4	4.9
Oral administration of 120 γ daily for 5 days, sacrificed afterward.	3.6	2.6
Placed on thiamine deficient diet for 7 days before study, sacrificed at 2 hrs. after oral administration of 50 γ.	7.1	3.1
Placed on thiamine deficient diet for 8 days before study, sacrificed at 50 minutes after oral administration of 240 γ.	18.4	16.1
Placed on thiamine deficient diet for 7 days before study, sacrificed at 1 hour after subcutaneous injection of 240 γ.	24.0	13.6
ditto	18.2	9.4
ditto	15.1	14.0
ditto	17.6	16.5

It was demonstrated that smaller amount of free thiamine resulted from TAD than from thiamine. It means that more esterification results from TAD administration than from thiamine administration.

DISCUSSION

The fact deserves attention that allithiamine and its homologue are more active on growth of animals than thiamine. As to the reason why it occurs, there is no evidence in favor of any difinite conclusion, although the fact that TAD exceeds in its rate of permeation into tissue seems to account greatly for this fact.

Since the intestinal uptake of allithiamine increases proportionally to the amount of intake, it is possible for us to make the blood level of thiamine in a subject attain any arbitrary value as we wish. Thus it is possible now to apply large doses of B_1 vitamin per os for therapeutic purpose. It is expected that the oral use of allithiamine may replace the injection of B_1 vitamin, releasing patients from pain, physicians from trouble.

Although no scientific explanation has been available on the widely prevailing custom to eat garlic as tonics among civil lives, there are ample evidences to contend that an effective approach will be made to this question from the study of allithiamine. Further report on this problem is contemplated.

SUMMARY

The results of our study on the effect of allithiamine and its homologue on experimental animals and human subjects may be summarized as follows:

1. Allithiamine and its homologue are less toxic than thiamine.
2. Allithiamine and its homologue are more active on growth of rats than thiamine.
3. Allithiamine and its homologue are curative effect on thiamine deficiency in experimental animals.
4. Allithiamine and its homologue are absorbed from intestine far more easily than thiamine. Intestinal uptake increases proportionately to the amount of oral intake.
5. Allithiamine and its homologue cause a marked elevation in blood level of thiamine in animals and human subjects.
6. Phosphorylation takes place on allithiamine more easily than thiamine.

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